UNIVERSITY OF ALBERTA

# MODULATING EFFECT OF DIETARY RHUBARB STALK POWDER ON CHOLESTEROL DEGRADATION

BY

VINTI GOEL ©

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The undersigned certify that they have read, and recommend to the faculty of Graduate Studies and Research for acceptance, a thesis entitled MODULATING EFFECT OF DIETARY RHUBARB STALK POWDER ON CHOLESTEROL DEGRADATION submitted by VINTI GOEL in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in NUTRITION AND METABOLISM.

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# ABSTRACT

Rhubarb (*rheum rhaponticum*), a cold season and hardy crop is vastly underutilized in Canada. Its stalks were utilized to prepare a dry powder (RSP), which was found to contain 74% dietary fiber, on a dry weight basis, with 66% insoluble and 8% soluble fiber. The potential ability of RSP as a hypocholesterolemic agent was investigated through the following objectives :1) to establish its cholesterol-lowering effects in experimental animals and humans; 2) to investigate the underlying mechanism(s) of its hypocholesterolemic action by determining : a) its ability to bind bile salts *in vitro* b) its influence on the bile salt excretion and on biliary bile salt pool in normocholesterolemic and hypercholesterolemic mice c) its role in bile acid synthesis through the activity and mRNA abundance of cholesterol 7$\alpha$-hydroxylase. Further, a study was also undertaken to examine, if RSP because of its oxalic acid content, has any untoward effect on the bioavailability of calcium.

Supplementation of RSP at a 5% level in semi-synthetic diet of hypercholesterolemic C57BL/6J mice resulted in significant reductions of plasma (-13%) and hepatic total-cholesterol (-34%) concentrations. The cholesterol-lowering response was specific to the LDL fraction in plasma ($P<0.05$) and the content of cholesteryl esters in the liver. Subsequently, a longitudinal clinical trial was undertaken to test if the response observed in experimental animals could be extrapolated to humans. Ten hypercholesterolemic men, not on any lipid lowering medications, were assigned to consume 27 g of RSP providing 20 g of dietary fiber / d, for 4 weeks. In parallel with the cholesterol-lowering effects of RSP in experimental animals, a significant decrease in total cholesterol ($p<0.05$) with specific reduction in LDL-cholesterol concentrations was

observed. These effects appeared to be a reflection of RSP intake since : a) the cholesterol levels of the subjects after 4 weeks of wash out were similar to preintervention values ; b) no appreciable change in dietary intakes with the exception of dietary fiber was observed between the onset of the study and at the end of RSP intervention and ; c) all ten study subjects displayed a declining trend.

Having established the cholesterol-lowering effects of RSP, its ability to bind bile salts was tested *in vitro*. Comparison with various fibers including wheat bran, rice bran, corn bran and cellulose, revealed that RSP had a stronger ability to bind both conjugated and unconjugated bile salts, and bound 11 and 2.5 fold more bile salt than cellulose and wheat bran, respectively. In agreement with the ability of RSP to sequester bile salts *in vitro*, the fiber intake at 5% dietary levels in C57BL/6J mice promoted the fecal loss of bile salts ($p<0.05$) in both the normocholesterolemic as well as hypercholesterolemic animals. This response was accompanied by a reduced gall bladder pool size of bile salts. The changes in the bile salt excretion were associated with an increased activity as well as mRNA abundance of hepatic microsomal cholesterol 7α-hydroxylase.

In view of the fact that RSP contains an appreciable amount of oxalic acid, the possible interaction between the fiber source and calcium bioavailability was examined. According to a balance study in rats, no detrimental effect of RSP on calcium bioavailability, up to its 5% level in the diet, was observed.

The results signify the potential use of RSP for the management of hypercholesterolemia. The ability to bind bile salts, and consequently to increase the bile salt excretion and therefore leading to an induction of cholesterol 7α-hydroxylase may account, at least in part for the hypocholesterolemic action of RSP.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| RSP | Rhubarb Stalk Powder |
| SCFA | Short chain fatty acids |
| HMG-CoA | Hydroxymethyl glutaryl-CoA |
| NADPH | Nicotinamide adenine dinucleotide phosphate (reduced form) |
| CE | Cholesteryl esters |
| TG | Triglycerides |
| CEH | Cholesterol ester hydrolase |
| LDL | Low density lipoprotein |
| VLDL | Very low density lipoprotein |
| HDL | High density lipoprotein |
| IDL | Intermediate density lipoprotein |
| LTP | Lipoprotein transfer protein |
| LCAT | Lecithin : acyltransferase |
| ACAT | Acyl-CoA : cholesterol acyltransferase |
| W/H | Waist to hip ratio |
| SAS | Statistical analysis system |
| ANOVA | Analysis of variance |
| SEM | Standard error of means |
| mRNA | Messenger ribonucleic acid |

# CHAPTER 1

## *INTRODUCTION*

Hypercholesterolemia is a major risk factor for coronary heart disease (CHD). In North America as many as 60% of men aged 20-50 years have cholesterol values above 200 mg/dL (5.2 mmol/L), indicating that they are hypercholesterolemic (Assan, 1987). One quarter of this population is hypercholesterolemic because of predisposition, however, the remaining three quarters respond to dietary intervention. Diet recommendations made for this population include restriction of total fat intake to < 30% of total calories, saturated fat intake to < 10% calories, cholesterol intake to less than 300 mg/day and increasing the intake of dietary fiber (Goodman, 1988). Diets high in dietary fiber have been shown to be valuable, specifically to combat mild to moderate hypercholesterolemia, where drug therapy can not be initiated, and reductions up to 10% in plasma total and LDL-cholesterol concentrations have been reported with various fiber sources. (Basu & Ooraikul, 1995; Hunt et al., 1993). Dietary fiber, however, is a generic term that includes a wide variety of substances that differ widely in physiochemical properties (Kritchvesky, 1988). Therefore, the physiological effects of fiber vary from source to source. The properties of a fiber that have been related to its ability to lower plasma cholesterol concentrations include solubility, fermentability, viscosity and bile acid binding ability. Most studies involving hypocholesterolemic effects of dietary fibers have been carried out using either soluble ones such as pectin and psyllium, or insoluble fibers such as cellulose.

The focus of this thesis was to investigate the cholesterol-lowering potential of fiber, prepared from the stalks of rhubarb plants. This new fiber source is described as rhubarb stalk powder (RSP). It contains 66% insoluble and 8% soluble fiber, on dry weight basis, in association with some other components such as proteins, ash and some organic acids such as oxalic and malic acids. This fiber source at 5% dietary level for 4 weeks has been reported to result in significant decreases in plasma and liver cholesterol concentrations in hypercholesterolemic mice (Basu et al., 1993). This preliminary study has thus signified the value of RSP as being a potential hypocholesterolemic fiber source.

The objective of this thesis was to examine the cholesterol - lowering ability of the new fiber source in both animals and humans. Since RSP was found to contain a considerable amount of oxalic acid, which is thought to affect calcium absorption, its effect on the bioavailability of calcium was also examined. Further, to elucidate the probable mechanism of hypocholesterolemic action of RSP, this study investigated its influence on the metabolism of cholesterol, with particular references to its degradation to bile acids, their synthesis, secretion, elimination and reabsorption.

# 1 *DIETARY FIBER*

Dietary fiber is defined as plant polysaccharides and lignin which are resistant to digestive hydrolysis by the enzymes of the upper intestinal tract in man (Trowell et al., 1976).The therapeutic benefits of dietary fiber in diseases of gastrointestinal tract, obesity, diabetes and cardiovascular diseases are well recognized. The thrust of this thesis is to study the influence of dietary fiber on cholesterol metabolism, which has been implicated in its therapeutic effects on cardiovascular disease. The physiochemical properties of a fiber that have been held responsible for its cholesterol-lowering effects include its solubility, viscosity, fermentability and binding ability (Hunt et al., 1993).

## *1.1 Physiochemical properties of dietary fiber and plasma cholesterol*

### *1.1.1 Solubility*

Analytically dietary fiber is classified by its solubility (Anderson, 1986). Insoluble fiber consists of structural or matrix fibers, such as lignin, cellulose, and some hemicelluloses, whereas soluble fiber consists of gel-forming substances such as pectins, gums and mucilages (Silk, 1989). The hypocholesterolemic effects of a dietary fiber have been linked more with soluble than insoluble fibers (Kashtan et al., 1992; Ebihara & Scheeman, 1989, Tinker et al., 1994; Horton et.al., 1994; McCall et al., 1992a; Fernandez, 1995). Since solubility of a fiber usually accompanies viscosity and /or fermentability, it has been suggested that these properties of soluble fibers might contribute to their cholesterol lowering effects (Topping, 1991).

### *1.1.2 Viscosity*

Viscosity, or gel forming capacity is related to fiber's ability to adsorb water and to form a gelatinous mass (Eastwood & Passmore, 1983). Soluble fibers such as pectins, mucilages and, to a limited extent hemicelluloses, elicit high water holding capacity and raise the viscosity of gastrointestinal contents. This property of fibers has important physiological consequences. This delays gastric emptying and creates a viscous environment in the intestine, thereby causing an increase in the thickness of unstirred water layer, which has been known to hinder the diffusion of macronutrients and therefore, slows absorption. The known consequences are improved glucose tolerance, flattened glucose response, and reduced insulinemia (Jenkins et al., 1978; Begin et al., 1989). Viscosity has also been suggested to interfere with lipid absorption by slowing down the diffusion of lipid containing micelles (Gallaher et al., 1993a; Gallaher et al., 1993b) but, the evidence to date is not very convincing. Although, recent reports by Gallaher et al (1993a, 1993b), suggest that viscosity of intestinal contents is strongly related with plasma cholesterol reduction, the earlier studies involving methylcellulose of different viscosities (Topping et al., 1988) and chitosans (Sugano et al., 1988) did not find this association.

### *1.1.3 Fermentability*

Although resistant to the action of human upper intestinal enzymes, passage of fiber through the ileocecal valve exposes fiber to bacterial enzymes that selectively degrade many of its components. The extent of fiber degradation in the colon depends on the nature of the colonic bacterial flora, and physical and chemical composition of the fiber.

Some fibers such as pectins, gums and mucilages can be degraded up to 100 % (Roberfroid, 1993). The products of fiber fermentation include the following compounds: carbon dioxide, methane, hydrogen, water and short chain fatty acids (SCFA), which include formate, acetate, propionate and butyrate. The SCFAs are largely absorbed via the portal blood and reach both the liver and peripheral tissues (Roberfroid, 1993). Some reports have indicated the systemic effects of SCFAs on both hepatic and peripheral metabolism of carbohydrates, lipids and cholesterol (Roberfroid, 1993). Effects of SCFAs on cholesterol metabolism are thought to be mediated by acetate and propionate (Bridges et al., 1992). The evidence for propionate is greater than for acetate (Chen et al., 1984), since oral propionate resulted in lowering of plasma cholesterol concentrations by inhibiting hepatic cholesterol synthesis when fed to animals such as rats and pigs (Illman et al., 1988; Boila et al., 1981). Although propionate inhibited liver cholesterol synthesis in isolated hepatocytes, the concentration required was several fold higher than those found *in vivo* (Illman et al., 1988). Also in humans, the effects of oral propionate on plasma cholesterol concentrations are uncertain (Venter et.al., 1990; Todesco et al., 1991, Wolever et al., 1996), thus making it unlikely as a mediator of hypocholesterolemic effects of a fiber.

### *1.1.4 Binding ability*

A number of organic materials such as bile acids, other steroids, various toxic compounds, divalent cations, and some bacteria may be bound to a fiber as it passes along the gastrointestinal tract (Kritchevsky, 1995). Adsorption of bile salts in a number of *in* *vitro* studies has been well documented (Kritchevsky & Story, 1974; Story & Lord, 1987) and is dependent on the composition of the fiber, the chemistry of the sterols, and the pH and the osmolarity of the surrounding medium. Lignin is the most potent bile acid sequestrant (Story & Lord, 1987). Pectin and other acidic polysaccharides also sequester bile acids (Story & Lord, 1987). Cellulose, in contrast, has little bile acid binding ability (Story & Kritchevsky, 1976). Bile salt structure is also an important determinant of the fiber's affinity for the bile salts (Vahouny et al., 1980). Dihydroxy bile salts conjugated with taurine, such as taurochenodeoxycholate, bind with greater affinity than either trihydroxy or glycine conjugated bile salts such as glycocholic acid (Vahouny et al., 1980). The pH of the surrounding medium also influences the binding with an increase in pH causing desorption of the bile salt bound to the fiber (Floren & Nilsson, 1987). The bile salt adsorption capacity of a fiber is measured *in vivo* as the ability to increase fecal bile salt excretion and has been related with cholesterol lowering effects of certain fibers. Although lignin is a potent bile acid sequestrant both *in vitro* as well as *in vivo* (Gallaher & Schneeman, 1986), it does not seem to be used as a hypocholesterolemic agent. This suggests, that this property too in itself is not sufficient to bring about significant reductions in plasma cholesterol concentrations.

It is, therefore, plausible that multiple properties might be involved in the hypocholesterolemic response to a fiber source and since fibers vary considerably in these physiochemical properties, the ability to reduce cholesterol varies with different fibers (Table 1.1). However, a consistent response observed with fibers that are effective in lowering cholesterol is the ability to increase fecal bile salt excretion, though other properties such as viscosity or fermentability are also desired.

Most studies examining the cholesterol-lowering effects of dietary fibers have been carried out with purified fiber sources such as pectin, psyllium, guar gum or cellulose. Data related to the effects of mixed fibers, (soluble + insoluble) is sparse. Such fiber sources represent a complex mixture of fiber types and may also contain different amounts of associated nutritive and non-nutritive materials such as proteins, phytosterols, minerals; some of which may influence sterol metabolism (Potter et al., 1993; Ling & Jones, 1995; Govers et al., 1994; McIntosh et al., 1991). The focus of this thesis was to establish cholesterol-lowering efficacy of a mixed fiber source prepared from the stalks of rhubarb plant, an underutilized crop.

*Table 1.1* ***Physiochemical properties of some fibers in relation to their cholesterol lowering effects***

| Fiber | Solubility | Viscosity | Fermentability | Bile acid binding ability | Fecal bile salt excretion | Cholesterol lowering effects | Reference |
|---|---|---|---|---|---|---|---|
| Pectin | Yes | Yes | High | Yes | Yes | Yes | Moundras et al., 1994 |
| Psyllium | Yes | Yes | High | ND | Yes | Yes | Miettinen & Tarpila, 1989 |
| Cyclodextrin | Yes | Low | Moderate | ND | Yes | Yes | Favier et al., 1995 |
| Cellulose | No | No | Low | No | No | No | Ebihara & Schneeman, 1989 |
| Lignin | No | No | No | Yes | Yes | No | Gallaher & Schneeman, 1986 |
| Wheat bran | No | No | Low | No | No | No | Kashtan et al., 1992 |
| Corn bran | Mixed | Low | Low | ND | Yes | Yes | Kishimoto et al., 1995 |
| Soy Fiber | Mixed | Low | Moderate | ND | ND | Yes | Lo et al., 1986 |

ND : Not determined.

# 2 RHUBARB

## 2.1 History

Rhubarb's history as a highly popular laxative drug and a general tonic dates back to about 2700 BC in China and the Mediterranean region, where roots of the plant were used for medicinal purposes. The parent species of present day commercial rhubarb is accredited to southern Siberia and the regions of the Volga. It is generally believed that camel caravans crossing the deserts and mountains from the Far East carried what was then the highly prized medicinal herb, rhubarb. Later, natives along the Volga discovered a plant similar to the "great medicine of China" and use of leaves spread over Europe (Boswell, 1949). The rhubarb of the garden type was introduced into Europe from the East by Vasco Da Gama in 1497. It was cultivated at Pauda, Italy, in about 1608 and some 25 to 30 years later, seeds of it were obtained for planting in England, but not until 1778 (Rowland, 1969) was it definitely recorded as a food plant and was used for making tarts and pies. Rhubarb farming in North America began in 1900 in forcing houses, cold-cellars, and hot-houses and the major production came from the Midwest United States, i.e., from Michigan, Indiana, and Illinois (Unpublished observation).

## 2.2 Botany

Rhubarb belongs to a buckwheat family, *Polygonaceae*, and to the genus *Rheum*, which contains perhaps 25 species. The common cultivated rhubarb belongs to the *Rheum rhaponticum.* Buckwheat and rhubarb are the only important food plants of the family grown in North America. The sourness of the juice is the characteristic of the family (Rowland, 1969).

Rhubarb is a herbaceous perennial plant, the underground portion of which consists of large, fleshy and somewhat woody rhizomes and a fibrous root system. The plant can attain a height up to 4 to 6 feet and contains a hollow stem with conspicuous nodes and relatively small leaves. The plant bears numerous small, greenish yellow flowers. Botanically, rhubarb is a vegetable, however, in use it is considered a fruit, since its principal use in the home is that of other types of fruits (Rowland, 1969).

### *2.3 Cultivation*

The rhubarb crown and rhizomes are resistant to cold and dry conditions and require temperatures below $4^0$C for the production of leaves and petioles. Foliar growth declines when day time temperature exceeds $26^0$C. The plant thrives best in regions where the crowns remain frozen all winter and hence grows during the spring and summer. The plant can also thrive in regions where winters are mild and summers are rainless, rhubarb then grows during the winter and early spring and remains dormant during the summer. At relatively low temperature for growth, the stalks develop the pink color, while at high temperatures, the green color predominates (Hauster, 1989).

Rhubarb can thrive on almost any type of soil from peat or sand to heavy clay, provided it is well drained. It is tolerant to wide range of soil pH but does best at pH between 6.0 to 6.8. Once planted, rhubarb remains in production for 10-15 years if properly maintained. Rhubarb has little problem with pests and diseases and can yield between 21-33 tons of stalks per acre. Rhubarb has also been grown in forcing houses, cold cellars or hot houses. For forcing, the rootstalks are exposed to the winter weather for two weeks. Then they are covered with barrels. The young leaf stalks develop rapidly with pale red color and have yellowish instead of green leaf blades.

The edible portion of the plant is the very elongated and thickened leaf stalk. The length, width and color of leaf stalk varies greatly among varieties. Stalks that are fresh, firm, crisp, tender and either red or pink in color are considered to be of good quality. They provide a product which is tender and free from strings when cooked. Stalks of the rhubarb that are well colored are usually well flavored. Forced rhubarb is usually lighter in color than field grown rhubarb. The younger stems on which the leaves are not fully grown are usually the most tender and delicate in flavor. Old rhubarb or that which has grown too long before being pulled may be pithy, rough and stringy. Tenderness and crispness can be tested by puncturing or snapping the stalk (Rowland, 1969).

The stalks of field grown rhubarb tend to be rich, dark red in color with coarse, green foliage and a very tart flavor. They are sold with leaves attached or removed. Hothouse rhubarb stalks tend to be generally light pink with small leaves, and usually are almost stringless. They also exhibit mild flavor (Rowland, 1969).

The leaf blades of rhubarb contain a high content of oxalic acid mainly in soluble form and hence can be quite toxic. Thus, they are not used for food purposes. In the stalks oxalic acid is present in smaller amounts and is largely in an insoluble form and therefore are considered safe for human consumption (Foust, 1992).

### *2.4 Varieties*

There are more than 100 varieties of rhubarb, but only a few are grown commercially. Varieties currently used for commercial production can be broadly classified as green, pink, or red petiole types. Green varieties have petioles that are almost completely green when grown in the field. The petioles of red types are almost completely red, while pink types vary in shade from pink to red. Among varieties it has been generally observed that the more red colored the stalks are, the less vigorous is the plant. Red types average 50 to 75% the yield of pink and green types. Pink types are most commonly grown for processing. Besides color and yield, absence of seed stalk production is main characteristic for which varieties are selected. Little or no seed stalk production is desired, since seed stalks interfere with harvest and inhibit production of leaves and petioles. The following is the list of some varieties and observations made on them in southern Alberta at the Horticultural Research Center in Brooks (Unpublished observation):

1. German Wine - the most vigorous variety that produces very large, green stalks; suitable only for juicing and wine products.
2. MacDonald - moderately red colored stalks, very good vigor, excellent for pie filling.
3. Early Sunrise - very similar to MacDonald but vigor is moderately less.
4. Valentine - bright red color make this variety very attractive, vigor is lacking somewhat.
5. Cherry red - bright red colored stalks; the plant lacks vigor.
6. Canada red - good vigor along with very strong red colored stalks.

The Clarksville (Michigan) Horticultural Experiment Station, a branch of Michigan State University, claims to have the world's largest accumulation of rhubarb varieties at a single location. Some sixty varieties have been planted in this center since 1979, in an effort to evaluate desirable characteristics such as yield, sugar content, acidity and color (Unpublished observation).

### *2.5 Production in North America*

The bulk of the rhubarb crop in North America comes from West Coast: Washington, Oregon and California, accounting for more than three quarters of commercial rhubarb grown in the United States. The state of Washington alone produces up to nearly 60% of the total crop (which is slightly less than 20 million pounds) and has nationwide marketing. The eastern cities of Boston, New York, and Montreal account for the largest usage. Most of the rhubarb production are frozen by processing companies for use in the making of pies, jellies or jams. Rhubarb is also processed to make rhubarb wine and 'Rhubarb Frost', a frozen drink made from rhubarb, orange, strawberry and lemon juice concentrates. A small amount is sold fresh, mostly in roadside markets and local stores (Unpublished observation).

Rhubarb has not been an important commercial crop. In Canada, a substantial scope exists for its increased production because the climatic conditions do not interfere with its growth. In Alberta, there is only one commercial grower who used to supply fresh rhubarb stalks to a winery in northern Alberta until it stopped making rhubarb wine recently (Unpublished observation). Small growers supply fresh rhubarb stalks to the market during summer and fall, while in winter rhubarb stalks are available in frozen form mainly from the US Since, rhubarb is a hardy and a cold resistant crop, the long and cold winter does not adversely affect its foliar growth. The moderately alkaline soil, as characteristic of southern Alberta are best suited to its requirements. Therefore, potential scope exists for the increased production and commercialization of the crop.

Rhubarb stalks not only serve as a fair source of nutrients (Table 1.2), the structure and composition of its fiber warrant its large commercial potential. Rhubarb stalks contain long strands of fibers made of bundles of helical coils that possess the ability to entrap water, a characteristic unique to only certain vegetable fibers such as celery and asparagus (Unpublished observation). The fiber has fractions of both soluble and insoluble types of fibers. Thus, in addition to be potentially useful as a filler or bulking agent in food products, the fiber also possesses the ability to confer health benefits of both the types of fibers. Although many vegetable fibers such as corn, soy etc. have fractions of both soluble and insoluble fibers, these vegetables are already in great demand both fresh and processed, therefore it is not economically expedient to use them just for fiber. Rhubarb is still vastly underutilized, commercially, or industrially, therefore substantial efforts have been made to extract and concentrate dietary fiber from rhubarb stalks without altering much of its physical or chemical properties. Techniques have been developed for the extraction of both, the wet fiber in the form of long and fine strands, as well as dried and ground fiber, from rhubarb stalks.

***TABLE 1.2 Composition and nutritional value of fresh rhubarb stalks***

| Components | Rhubarb stalks (100g) |
|---|---|
| Energy (Kcal) | 20 |
| Moisture (%) | 93.8 |
| Protein (g) | 0.4 |
| Fat (g) | 0.1 |
| Carbohydrate including dietary fiber (g) | 4.8 |
| Calcium (mg) | 60 |
| Phosphorus (mg) | 20 |
| Iron (mg) | 0.5 |
| Potassium (mg) | 299 |
| Magnesium (mg) | 13 |
| β-Carotene (μg) | 80 |
| Thiamin (mg) | 0.04 |
| Riboflavin (mg) | 0.03 |
| Niacin (mg) | 0.2 |
| Oxalic acid (mg) | 4.8 |
| Vitamin C (mg) | 16 |

Leung et al., 1972

Studies involving both wet and dry rhubarb fiber are being carried out to test its potential ability to serve as a filler, bulking material and texture modifier in bakery and meat products (Unpublished observation). The fiber has been successfully incorporated into meat and vegetarian patties and the potential ability of the fiber to be incorporated into meat and vegetarian jerkies is also being tested. Nutritional studies involving rhubarb stalk powder (RSP) were also undertaken to test its potential value as a dietary fiber supplement for health benefits.

In a preliminary study, the potential hypocholesterolemic action of RSP was determined in hypercholesterolemic mice. Feeding these animals a semi-synthetic diet containing 5% RSP for 4 weeks resulted in significant reductions of both plasma and liver cholesterol concentrations as compared to mice fed a diet containing 5% cellulose (Basu et al., 1993). The new fiber source also led to an increase in HDL-cholesterol to total cholesterol ratio. The effects were not related to the suppression of hepatic cholesterol synthesis.

The intriguing results from this preliminary study, encouraged studies to further explore the biological potential ability of RSP. Studies were thus, undertaken to examine its cholesterol lowering effects in animals and humans and, to elucidate the probable mechanism of its hypocholesterolemic action. Since RSP was found not to inhibit hepatic cholesterol synthesis (Basu et al., 1993), the focus of the present study was to investigate the effects of RSP on cholesterol metabolism, with particular references to cholesterol degradation to bile acid synthesis, elimination and reabsorption.

## *3 OVERVIEW OF CHOLESTEROL METABOLISM*

Cholesterol is found in all mammalian cells and is confined largely to the plasma membrane. The cholesterol content of membranes is tightly regulated, and cells undergoing mitosis retain all the enzymes necessary for cholesterogenesis. Although the functions of cellular cholesterol are still incompletely understood, it is known that cholesterol in membranes increases their viscosity and decreases their permeability to small water-soluble molecules. Despite its essentiality in cells, the accumulation of free and esterified cholesterol in and between the cells within the vascular bed is the major cause of pathogenesis of atherosclerosis. An increased level of plasma cholesterol (particularly LDL-cholesterol) is considered an independent risk factor for this disease. A number of cholesterol-lowering drugs such as bile acid sequestrants, HMG-CoA reductase inhibitors and fibric acids are currently available (Gamble, 1994). However, owing to their various side effects, diet therapy is the mainstay in the treatment of moderate hypercholesterolemia. Dietary fiber is one of the dietary components, which is thought to participate in lowering cholesterol status. To understand the role of dietary fiber in the management of hypercholesterolemia, it is essential to understand basic cholesterol metabolism.

### *3.1 Biosynthesis of cholesterol*

The precursor pool of cholesterol synthesized in mammalian cells is the acetyl-CoA present in the cytosol (Fielding & Fielding, 1991). The first step involves the formation of acetoacetyl-CoA from two acetyl units, a reaction which is catalyzed by a specific thiolase. The reaction is driven to completion by the subsequent condensation of acetoacetyl CoA with another acetyl-CoA unit to form hydroxymethylglutaryl-CoA (HMG-CoA) catalyzed by HMG-CoA synthase. HMG-CoA is converted to mevalonic acid by HMG-CoA reductase in the presence of NADPH. The reductase is a microsomal enzyme whose activity is generally rate limiting in the reaction sequence leading to cholesterol. The enzyme is regulated by phosphorylation, diurnal cycle, levels of cellular cholesterol (Osborne et al., 1985) and oxygenated cholesterol derivatives which may be formed in the cell during cholesterol metabolism such as 27-hydroxycholesterol (Esterman et al., 1983).

Mevalonic acid forms isopentenyl pyrophosphate by phosphorylation and loss of $CO_2$ unit by specific kinases. Six units of isopentenyl pyrophosphate are condensed to give presqualene pyrophosphate, which is converted in the presence of NADPH to squalene, with the loss of pyrophosphate. Squalene forms lanosterol by reactions catalyzed by a mixed function oxidase (squalene epoxidase) and 2,3-oxidosqualene:lanosterol cyclase. Finally lanosterol is converted to cholesterol through a series of reactions catalyzed by microsomal enzymes. The relative rates of cholesterol synthesis vary among different tissues (Fielding & Fielding, 1991). Liver plays a major role in the synthesis of cholesterol, while the contribution of intestine is also important. These tissues secrete cholesterol into the plasma in lipoprotein form.

### *3.2 Plasma Lipoproteins*

Cholesterol, both free and esterified, is secreted in lipoprotein form from intestinal mucosal cells into the mesentric lymph ducts and thence into the plasma, and from hepatocytes directly into the plasma. Lipoproteins are most commonly classified on the basis of their floatation density and contain a core (lipids) and a coat domain (apoproteins). On the basis of core, they are divided into two categories :

Triglyceride - rich lipoproteins (Chylomicrons and VLDL)

Cholesteryl ester - rich lipoproteins (LDL, HDL)

### *3.2.1 Chylomicron metabolism*

Chylomicrons function in the transport of exogenous triacylglycerols. They are synthesized in the small intestine and carry primarily triglycerides and some cholesterol. They are the largest particles found in the circulation and are rapidly metabolized upon entry into circulation with a half-life of about 15 min. Nascent chylomicrons contain apo B-48 and apo A-1 (Fielding & Fielding, 1991). Their catabolism proceeds by first initial rapid binding to lipoprotein lipase (LPL) on the endothelial surface of blood capillaries in adipose tissue, cardiac, skeletal muscle and other tissues. LPL hydrolyses most of the triglycerides of the chylomicrons. As a result these particles shrink in size and form chylomicron remnants. The remnants acquire apo-E from circulating HDL and are very rich in cholesteryl esters. They proceed to the liver, where they are taken up by receptor mediated endocytosis which recognizes apo-E (Fielding & Fielding, 1991).

### *3.2.2 VLDL metabolism*

VLDL functions in the transport of endogenous triglycerides. The initial step of VLDL metabolism is the same as chylomicron metabolism i.e., interaction with LPL. Thereafter they are converted to intermediate density lipoproteins (IDL), and similar to chylomicron remnants are very rich in cholesteryl esters and contain apo B-100 and E. Half of these IDL are taken up by the liver, whereas the other half is converted to LDL. LDL reacts with the apo B/E receptor present on the surfaces of most cells and its cholesteryl esters are interiorized (Fielding & Fielding, 1991).

In contrast to receptor mediated uptake of cholesteryl esters of lipoproteins by cells, free cholesterol content of lipoproteins is internalized directly via transfer to cellular plasma membranes. Once internalized esterified cholesterol is hydrolyzed by the enzyme cholesterol ester hydrolase (CEH) and free cholesterol also called as 'metabolically active pool' regulates cholesterol balance in the cell (Brown & Goldstein, 1986).

### *3.2.3 HDL metabolism*

Liver also secretes apo A-1 rich particles, called HDL, which function in the removal of cholesterol from the cells. HDL derives free cholesterol from cells in which the rate of cholesterogenesis is greater than required for the synthesis of new membranes. The mechanism by which tissues not secreting lipoprotein particles return cholesterol to the liver has been called reverse cholesterol transport. The cholesterol efflux from nonhepatic tissues into circulation is maintained predominantly by the esterification of cholesterol by LCAT (lecithin:cholesterol acyltransferase). The evidence for the role of LCAT comes from patients with a genetic deficiency of LCAT, in which free cholesterol accumulates in the cells (Fielding & Fielding, 1995). Once cholesterol is derived from the cells, a part of cholesteryl ester core of $HDL_2$ is exchanged for triglycerides by a lipoprotein transfer protein (LTP-1) from VLDL or LDL. Triglyceride rich $HDL_2$ particles are hydrolyzed by a hepatic lipase, the small and less dense HDL particles so formed are called $HDL_3$. $HDL_3$ is converted back to $HDL_2$ by acquiring apolipoproteins from VLDL and serves as a good substrate for LCAT. The rest of the HDL particles are metabolized in the liver (Fielding & Fielding, 1995).

## *3.3 Regulation of cholesterol balance in the cell*

### *3.3.1 LDL Receptor*

Cholesteryl esters are delivered to the cells by a receptor that specifically recognizes apo-B or apo-E, and is called the apo-B/E receptor. Therefore LDL and chylomicron remnants are its main substrates. The apolipoproteins bind to the receptor, the receptor-LDL complex is internalized by endocytosis and its components are degraded by lysosomal enzymes. The receptor is recycled back to the plasma membrane and reutilized. The levels of free cholesterol in the cell regulate the activity of the receptor, with high levels inhibiting its further synthesis, at the transcriptional level (Sudhof et al., 1987).

### *3.3.2 Cholesterogenesis*

The content of free cholesterol in the cell also regulates cholesterol synthesis by regulating the rate limiting enzyme HMG-CoA reductase both at the level of gene expression as well as enzyme activity (Osborne et al., 1985). HMG-CoA is also inhibited by several structural analogues of HMG-CoA that have been isolated from microorganisms, such as compactin and mevinolin, and also some plant phytosterols such as saponins and tocotrienols (Quershi et al., 1987).

### *3.3.3 Esterification of cholesterol (ACAT)*

Free cholesterol in the cell also favors the formation of intracellular cholesteryl esters synthesized by acyl-CoA:cholesterol acyltransferase (ACAT). ACAT is a microsomal enzyme and when rate of uptake of cholesterol by LDL-receptor exceeds rate of efflux by HDL, ACAT is induced (Fielding & Fielding, 1991). Since, ACAT regulates cellular levels of free cholesterol, the metabolic pool of cholesterol, an association between ACAT and LDL receptor has also been demonstrated (Rumsey et al., 1995). Increased activity of ACAT in macrophages has been linked with the development of foam cells (Ginsberg, 1991). In liver, higher levels of free cholesterol favor cholesterol degradation by regulating the enzyme cholesterol 7α-hydroxylase (Vlahcevic et al., 1992).

## *3.4 Cholesterol degradation*

The whole body cholesterol homeostasis is maintained by the liver. More than 75% of the newly synthesized cholesterol in the whole body is produced by the liver and it is the only organ capable of eliminating cholesterol from the body. Cholesterol is eliminated from the body by two major output pathways, cholesterol degradation to bile acids (bile acid biosynthesis) and biliary cholesterol secretion. The secretion of biliary cholesterol stays relatively constant. The bile acid synthesis pathway can account for up to 70% of cholesterol elimination from the body (Everson, 1992).

### *3.4.1 Bile acid biosynthesis*

Bile acid synthesis from cholesterol can occur via two pathways. One initiated by cholesterol 7α-hydroxylase begins in the endoplasmic reticulum of the hepatocytes and utilizes cholesterol as the substrate whereas the other initiated by sterol 27-hydroxylase occurs in mitochondria with the high activity in vascular endothelial cells, uses 27-hydroxycholesterol as the substrate (Javitt, 1994). In both the pathways the end products of metabolism are primary bile acids. Although, the physiological significance of 27-hydroxylase pathway in the maintenance of plasma lipid levels has recently been recognized in cholesterol 7α-hydroxylase knockout mice (Schwartz, 1996), cholesterol 7α-hydroxylase is the most active path when the enterohepatic circulation of bile acids is interrupted (Everson, 1992) and will be the focus of this discussion.

In man, the primary bile acids are cholic acid (3α, 7α, 12α-trihydroxy-5β-cholanic acid) and chenodeoxycholic acid (3α, 7α-dihydroxy-5β-cholanic acid). The biosynthesis of bile acids from cholesterol requires at least 14 different enzymes located in cytosol, microsomes, mitochondria and peroxisomes. Cholesterol 7α-hydroxylase is the first and most highly regulated and rate limiting enzyme in this pathway (Vlahcevic et al., 1992).

### *3.4.2 Cholesterol 7α-hydroxylase*

Cholesterol 7α-hydroxylase is a hepatic microsomal enzyme which belongs to cytochrome P450 family. It requires molecular oxygen, NADPH and cytochrome P-450 reductase for its activity. The gene for cholesterol 7α-hydroxylase has been cloned and its sequence determined from rat, human and hamsters (Jelinek et al., 1990; Cohen et al., 1992; Crestani et al., 1993). Analysis of 250 bp of the 5'-flanking regions of these genes revealed a high sequence identity of about 72 to 83%. The human gene is polymorphic, spans 10 kb, contains 6 exons and 5 introns, and has been mapped to chromosome 8q11-q12.

### *3.4.2.1 Regulation of cholesterol 7α-hydroxylase*

The activity of the enzyme is regulated principally at the transcriptional level and most important physiological regulators of cholesterol 7α-hydroxylase are hydrophobic bile acids, cholesterol, certain hormones, such as glucocorticoids and insulin and diurnal rhythm (Lavery & Schibler, 1993).

Convincing evidence suggests that the bile acids returning to the liver through enterohepatic circulation regulate cholesterol 7α-hydroxylase (Vlahcevic et al., 1992). Additionally, a bile acid responsive element (BARE) has been localized in the promoter region of the cholesterol 7α-hydroxylase gene (Chiang & Stroup, 1994). The potency of different bile acids as suppressers vary with their relative hydrophobicity. Taurine conjugated hydrophobic bile acids such as taurodeoxycholic acid have greater suppressive effects than either glycine conjugated or hydrophilic bile acids (Stravitz et al., 1993).

Although many studies have indicated an upregulation of cholesterol 7α-hydroxylase activity with the addition of cholesterol to the diet (Jelinek et al., 1990; Ramirez et al., 1994), the evidence is still not very convincing. A study on African green monkeys showed an opposite effect of dietary cholesterol (Rudel et al., 1994). Some recent data has indicated that dietary fats also have some role and have been shown to influence the regulatory potential of cholesterol on cholesterol 7α-hydroxylase gene expression (Cheema et al., 1997).

Hormonal responsive fragments on the promoter of the cholesterol 7α-hydroxylase gene also have been identified. Recent evidence indicate that the gene is activated by glucocorticoids and retinoic acid, and inhibited by insulin, phorbol esters and cAMP (Crestani et al., 1995).

### *3.4.3 Enterohepatic circulation of bile acids*

Prior to secretion from the hepatocytes, free bile acids (primary) are conjugated to either glycine or taurine, this conjugation increases their water solubility. Quantitatively, 98% of secreted bile acids are found in the conjugated form. They are secreted from the parenchymal cells of the liver into the common bile duct. In most mammals, the majority of bile is stored in the gall bladder and released upon stimulation by hormones, such as cholecystokinin, into the duodenum. It is here that bile acids act to solublize dietary fats and cholesterol, thereby facilitating the digestion of these nutrients by the lipases. During the passage through the gastrointestinal tract, primary bile acids undergo modification by numerous bacterial enzymes, resulting in the formation of secondary and tertiary bile acids (Russell & Setchell, 1992). As a result of this extensive bacterial modification, the diversity of bile acids in bile is quite large (Russell & Setchell, 1992).

Conservation of the bile acid pool is accomplished by the efficient reabsorption of bile acids from the gastrointestinal tract. Bile acids are absorbed throughout the length of the intestine by passive diffusion, however active uptake involving a specific $Na^+$-

dependent bile acid transport system has been characterized in the brush border membranes of ileal enterocytes (Kramer et al., 1992). Uptake varies with the bile salt structure. Conjugated bile acids are actively transported in the ileum. (Aldini et al., 1996). Then through the portal circulation bile acids return to hepatocytes through the transporters located on hepatocytes.

This enterohepatic circulation of bile acids is essential for the conservation of bile acid pool. Interruption of this circulation, as with bile acid sequestrants such as cholestyramine, results in rapid increase in bile acid synthesis causing up to 3-4 fold increase in the activity and mRNA abundance of cholesterol 7α-hydroxylase (Horton et al., 1994).

## *4 DIETARY FIBER AND CHOLESTEROL METABOLISM*

### *4.1 Dietary fiber and cholesterol synthesis*

To understand the precise mechanisms by which dietary fiber reduces hepatic cholesterol concentrations, a number of investigators have studied the effects of dietary fiber on cholesterol synthesis (Arjmandi et al., 1992b; Quershi et al., 1987; McCall et al., 1992a; Fernandez et al., 1994; Turley et al., 1991). Two purified fibers i.e., pectin and psyllium have been extensively studied using two different approaches i.e., either direct measurement of HMG-CoA reductase in the liver and in the intestine, or measurement of incorporation of [$^3$H] $H_2O$ into hepatic sterols. The results suggest that these fibers do not directly suppress hepatic cholesterol synthesis, since either no effect or even increased synthesis of cholesterol has been observed with these fiber sources. Some fibers such as oats and barley have shown suppressive effects on HMG-CoA reductase, due to the presence of phytosterols i.e., tocotrienols or saponins. These compounds, because of being a structural analog to HMG-CoA, have been shown to inhibit the activity of HMG-CoA reductase and therefore contribute to the hypocholesterolemic effects of these fibers (Qureshi et al., 1987). Studies with perfused livers (Illman et al., 1988) or isolated hepatocytes (Nishina & Freedland, 1990) have demonstarted a suppressive effect of propionate on cholesterol synthesis through the inhibition of HMG-CoA synthase. However, sterologenesis was inhibited solely when nonphysiological concentration (1mM) of propionate was present in the medium.

### *4.2 Dietary fiber and lipoprotein metabolism*

Although the LDL-cholesterol lowering effects of some of fibers are well documented, the mechanism is still not clear. In a study on hypercholesterolemic African green monkeys, McCall et al.(1992b) using simultaneous injections of $^{131}$I-labeled LDL and $^{125}$I-labeled VLDL demonstrated that psyllium husk reduced plasma cholesterol concentrations by decreasing LDL synthesis and had no effect on fractional catabolic rate of LDL. However, some studies have also indicated an activation of hepatic apo-B/E receptor with dietary fiber intakes in hypercholesterolemic animals (Fernandez et al., 1992; Fernandez et al., 1994; Horton et al., 1994). Changes in LDL density have also been reported with some fibers such as pectin (Fernandez et al., 1992), with fiber lowering the content of free and esterified cholesterol in LDL though increasing the concentration of triglycerides.

Effects of dietary fiber on microsomal ACAT activity though have not been extensively studied, but some reports have indicated a reduced activation of the enzyme with dietary pectin as compared to dietary cellulose in hypercholesterolemic animals (Basu et al., 1993; Fernandez et al., 1994).

### *4.3 Dietary fiber and bile acid metabolism*

Convincing evidence now exists to support the fact that the fibers which are effective as hypocholesterolemic agents exhibit the ability to influence bile acid metabolism. Increased excretion of bile acids has been reported with some fibers such as pectin, guar gum (Garcia-Diez et al., 1996; Moundras et al., 1994), corn fiber (Kishimoto et al., 1995), psyllium and oat bran (Arjmandi et al., 1992a). Increased activity as well as mRNA abundance of cholesterol 7$\alpha$-hydroxylase has also been reported with some fibers (Matheson et al., 1995; Horton et al., 1994; Fernandez, 1995). Matheson et al.(1994) also reported changes in bile acid pool size and bile composition with dietary psyllium and pectin in rats. These fibers although, increased the total bile acid pool but lowered the concentration of hydrophobic bile acids, thus optimizing the conditions for the activation of cholesterol 7$\alpha$-hydroxylase.

## *5. SUMMARY AND OBJECTIVES*

Evidence to date suggests that 'dietary fiber' includes a wide variety of substances that can vary widely in their physiochemical properties. They are valuable hypocholesterolemic agents but the effects cannot be generalized based on any single characteristic such as solubility or viscosity or fermentability or binding ability. Though the ability to increase fecal bile acid excretion is known to be essential for a fiber to have cholesterol-lowering effects, it too, in itself is not sufficient; other properties such as viscosity or fermentability seem to be required. Although a number of fiber sources have emerged as valuable hypocholesterolemic agents, the research has been focused predominantly on purified fibers. Mixed fibers or fibers as a part of foods have not been extensively tested, though their effects could be different from purified fibers. One mixed fiber source, rhubarb stalk powder, was tested in these studies for its potential as a hypocholesterolemic agent and its possible mechanism of action was determined. **It was hypothesized that RSP exerts an hypocholesterolemic action by binding with bile salts and consequently interrupting the enterohepatic circulation of bile acids.** This hypothesis was tested with following **objectives**

1. To establish the hypocholesterolemic effects of RSP in humans.
2. To investigate the underlying mechanism(s) of the hypocholesterolemic action of RSP by determining: a) the ability of RSP to bind bile salts *in vitro* and comparing its capacity with that of other fiber sources; b) the effects of RSP on bile salt excretion and on biliary bile acid concentration in normocholesterolemic and hypercholesterolemic mice; and c) the effects of RSP on bile acid synthesis through the activity and the mRNA abundance of cholesterol 7α-hydroxylase.
3. To examine if RSP, because of its oxalic acid content, has any untoward effect on the bioavailability of calcium.

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# CHAPTER 2

## Cholesterol lowering effects of rhubarb stalk fiber (RSP) in hypercholesterolemic men[1]

### *2.1 INTRODUCTION*

Elevated serum cholesterol concentrations, particularly low - density lipoprotein cholesterol is an important risk factor for coronary heart disease (American Heart Association, 1990). Considering the side effects of many cholesterol lowering drugs (Witztum, 1989), dietary modification has been identified by National Cholesterol Education Program as the primary treatment of choice for decreasing blood cholesterol levels (Goodman, 1988). Suggested dietary modifications include lowering the fat, especially saturated fatty acids and cholesterol content of the diet, and increasing the dietary fiber intake.

The hypocholesterolemic effects of dietary fibers vary according to the type of fibers. Gel forming or viscous fibers such as pectin (Jenkins et al., 1975; Assis & Basu, 1990; Fernandez et al., 1994), psyllium (Horton et al., 1994; Anderson et al., 1992) and guar gum (Jenkins et al., 1975; Anderson et al., 1992) have consistently shown decreases in plasma cholesterol concentrations in experimental as well as in clinical trials. In contrast, the cholesterol lowering properties have not been shown for insoluble fibers such as cellulose (Jenkins et al., 1975). However, the effects of mixed fibers containing portions of both soluble and insoluble fibers have been controversial. Some sources such as wheat bran (Kashtan et al., 1992; McIntosh et al., 1991) have depicted no effects at all, whereas some sources such as soy fiber (Sasaki et al., 1985; Lo et al., 1986), barley bran (Lupton et al., 1994) and corn bran (Hunningshake et al., 1994) have shown significant reductions in plasma cholesterol concentrations.

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[1] A version of this paper has been submitted to *Journal of the American College of Nutrition*

Dried and ground rhubarb stalks have been found to be a novel source of dietary fiber containing up to 74% dietary fiber with 66% insoluble and 8% soluble fiber on dry weight basis (Chapter 1). Preliminary study undertaken on experimental mice have indicated that rhubarb stalk powder (RSP) could be a valuable hypocholesterolemic agent since its supplementation at 5% level in the diets of hypercholesterolemic mice resulted in significant lowering of plasma and liver cholesterol concentrations. The present study was undertaken to test the efficacy of RSP in lowering plasma lipid levels in subjects with mild to moderate hypercholesterolemia.

## *2.2 SUBJECTS AND METHODS*

### ***2.2.1 Preparation of Rhubarb stalk powder (RSP)***

Rhubarb stalks of mixed varieties were obtained from small growers and food stores in Edmonton, Alberta. Both fresh and frozen stalks were used.

Figure 2.1 illustrates a processing procedure used for the separation and concentration of fiber from rhubarb stalks. Briefly, the stalks were cut into short pieces of 3 cm size. The cut stalks were either steam cooked for 10 minutes or microwaved at maximum power setting for >2 minutes. The softened stalks were then pressed with manual roller press to remove much of the juice. The pressed stalks were leached twice in about 10 times its weight of water at 70$^0$C for 15 min. and squeezed. The stalks were then dried in a fluidized bed dryer at 85$^0$C for about 1 h 40 min. The dry stalks were ground finely in a coffee grinder.

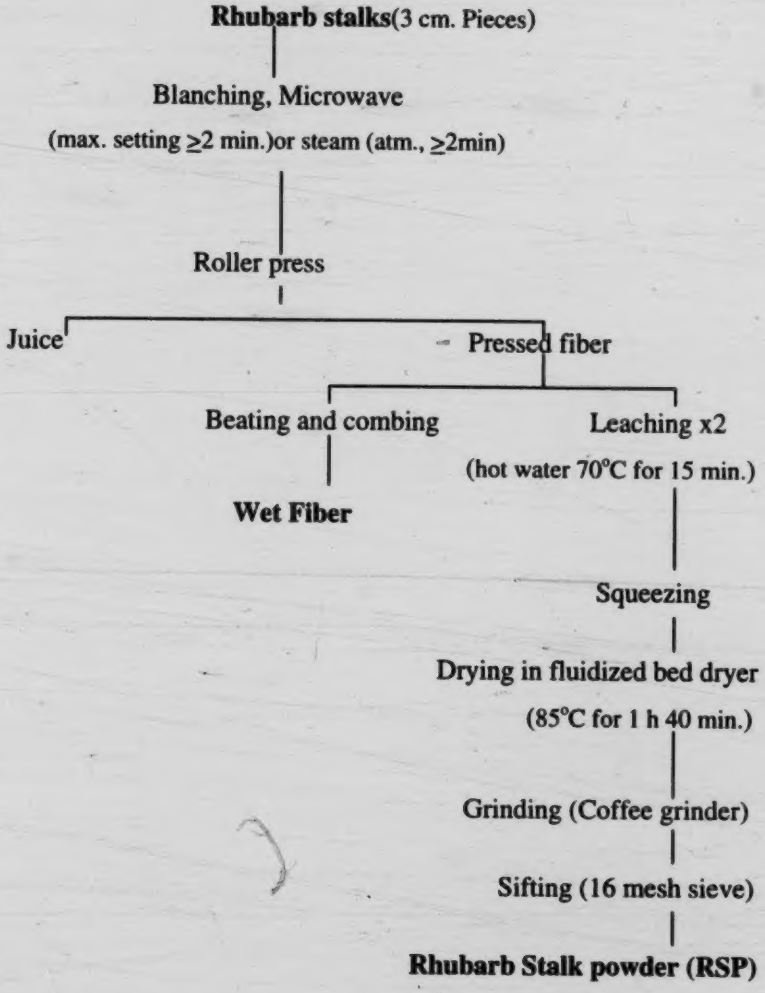
Rhubarb stalks(3 cm. Pieces)
Blanching, Microwave
(max. setting ≥2 min.)or steam (atm., ≥2min)
Roller press
Juice
Pressed fiber
Beating and combing
Wet Fiber
Leaching x2
(hot water 70°C for 15 min.)
Squeezing
Drying in fluidized bed dryer
(85°C for 1 h 40 min.)
Grinding (Coffee grinder)
Sifting (16 mesh sieve)
Rhubarb Stalk powder (RSP)

**Fig 2.1 Processing flowchart for rhubarb fiber**

### *2.2.2 Physiochemical properties of RSP*

The chemical composition of RSP was determined. The dietary fiber content of RSP was estimated using a method of Prosky et al (1988) and it was found to contain 74% of the total dietary fiber on dry weight (Table 2.1). Insoluble fiber contributed for the majority of the fiber (66%), though appreciable amounts of soluble fiber (8%) mainly as pectin were also present. RSP retained most of the oxalic acid of fresh stalks and it accounted for 5.7% of the dry weight. RSP contained appreciable amounts of other ingredients including calcium, protein and malic acid. Comparison with other fiber sources showed that the RSP contained about five times more total dietary fiber than oatmeal, with about the same concentration of soluble fiber.

The RSP elicited a water holding capacity of 1855 g/100 g dry powder and thus, absorbed almost 19 times its weight of water. The product had light brown color and was quite bland in aroma and taste.

### *2.2.3 Dosage*

RSP was sifted through a 16 mesh sieve and packed in small polyethylene packets, containing 27g of dry powder to be distributed to participants. Each subject received 30 packets, and asked to consume one packet per day with a beverage of the subject's choice.

**Table 2.1** *Chemical Composition of RSP*[1]

| | %, Dry weight |
|---|---|
| Protein | 5.6 |
| Ash | 5.6 |
| Calcium | 2.0 |
| Oxalic acid | 5.7 |
| Malic acid | 3.2 |
| Insoluble dietary fiber | 65.9 |
| Soluble dietary fiber | 8.2 |
| Pectin | 6.6 |
| Total dietary fiber | 74.1 |

[1] Values are means of 2-4 replicates.

### *2.2.4 Subjects*

Eighteen individuals volunteered for the study. All volunteers underwent a baseline evaluation, including physical examination and screening laboratory tests. Seven subjects could not participate in the study because of failure to meet the baseline inclusion criteria (Table 2.2) and one subject could not complete the study because of illness unrelated to the study. A total of ten apparently healthy men aged 30-60 years with a history of mild to moderate hypercholesterolemia were enrolled in the study.

**Table 2.2** ***Selection criteria for the subjects at study entry***

| Inclusion criteria | Exclusion criteria |
|---|---|
| Free living male individuals | Use of lipid-lowering drugs in past six months |
| Age 30-60 years | Body mass index $\geq$ 35 kg/m$^2$ |
| Plasma Cholesterol > 5.2 mmol/L | Plasma Triglycerides $\geq$ 5 mmol/L |
| Availability for regular follow ups | Known ailments such as renal, pulmonary, thyroid, diabetes or cardiovascular disease or any condition requiring diet modification |
| | On medications such as β-blockers |

The baseline characteristics of the subjects are given in Table 2.3. Participants were asked to keep their nutritional habits and level of activity constant and to avoid alcohol during the study. All subjects signed a consent form before the beginning of the experiment. This research was approved by the Human Ethics Committee of the University of Alberta.

**Table 2.3** ***Baseline characteristics of the subjects completing the study***[1,2]

| | Value |
|---|---|
| Age (y) | 44 ± 2.90 |
| Body mass index (Kg/$m^2$) | 27.9 ±2.80 |
| Waist / Hip ratio | 0.89 ± 0.01 |
| Plasma cholesterol (mmol / L) | 6.59 ± 0.07 |
| Smokers | none |

[1] Values are means ± SEM
[2] n = 10.

### *2.2.5 Experimental design*

The study lasted for 10 weeks and consisted of three experimental periods, that included a basal period of two weeks, a period of 4 weeks when 27g of ground RSP providing 20g of dietary fiber was taken each day, and a wash out period of 4 weeks when the RSP supplementation was withdrawn. Throughout the course of the study, all participants were called every week, especially, to check for compliance with the fiber intake, and were questioned about tolerance, acceptability and side effects attributable to RSP supplementation.

The effects of RSP on lipid profiles were determined by obtaining 4 overnight fasting blood samples from each subject during the course of the entire study. First two samples were taken at one week intervals before the start of the supplementation to establish the baseline lipid values. A third sample was drawn at week four of RSP supplementation and the final was obtained a month after the RSP withdrawal. Dietary intakes with particular references to total energy, lipids and dietary fiber, and anthropometric measurements [body mass index (BMI), and waist and hip ratio (W/H)] were obtained at baseline and at the end of dietary intervention.

### 2.2.6 *Biochemical analysis*

The blood samples were centrifuged at 600xg for 10 minutes to obtain serum and the separated serum was stored at -30°C pending analysis. The serum samples were analyzed for total cholesterol, HDL cholesterol and triglycerides by enzymatic kits obtained from Sigma Biochemical (Catalog # 352-3, 352-20 and 336-10 respectively). Serum LDL-cholesterol concentrations were calculated as described by Friedwald et al ((1972) using the following equation:

LDL-Cholesterol = Total Cholesterol - (HDL-Cholesterol + Triglycerides/5)

### 2.2.7 *Dietary intake*

Dietary interviews were conducted by a trained dietitian at baseline and at the end of the RSP intervention. The dietary intakes were assessed quantitatively using 2x24 h diet recalls accompanied by a detailed food frequency questionnaire. Each subject was asked to recall all food items including beverages over the previous 48 h period. Food frequency questionnaires were prepared by including all food groups and covering most of the food items available in local food stores. Food models constructed according to Nutrition Canada (Health & Welfare Canada, 1973) were used to estimate portion sizes. The daily nutrient intakes of total calories, protein, carbohydrates, saturated , monounsaturated and polyunsaturated fatty acids, cholesterol and total dietary fiber were calculated using nutrient composition tables (Watt & Merritt, 1975; United States department of Agriculture, 1976-1993) and averaging the estimates obtained by 2x24 h recalls and food frequency questionnaires.

### *2.2.8 Data Analysis*

The baseline values for serum lipids were calculated by averaging two values obtained before RSP supplementation. The statistical significance of differences in anthropometric measurements, nutrient intakes and serum lipids following fiber intervention was assessed by analysis of paired differences where each subject was compared to his own control values using SAS software. Differences were judged to be statistically significant if the associated P value was $< 0.05$ (Steel & Torrie, 1987).

## *2.3 RESULTS*

The RSP supplementation for 4 weeks had no effect on the BMI or W/H ratio of the subjects. Analyses of dietary intakes of nutrients at preintervention and postintervention by 2x24 h dietary recalls and food frequency questionnaires showed that the subjects maintained constant dietary intakes throughout the study (Table 2.4). RSP intervention caused a 2.5 times increase in the subjects average daily dietary fiber intake.

**Table 2.4** ***Daily nutrient intakes of the subjects***[1]

| Nutrients | Baseline[2] (n=10) | Final[3] (n=10) |
|---|---|---|
| Energy(Kcal/day) | 2486±13.48 | 2520±13.48 |
| Fat(g/day) | 87.70±0.62 | 87.53±0.62 |
| Protein(g/day) | 88.45±0.78 | 89.16±0.78 |
| Saturated fatty acids(g/day) | 25.03±0.25 | 24.83±0.25 |
| Monounsaturated fatty acids(g/day) | 30.83±0.13 | 30.75±0.13 |
| Polyunsaturated fatty acids(g/day) | 16.20±0.03 | 16.24±0.03 |
| Cholesterol(mg/day) | 207.94±1.30 | 211.01±1.30 |
| Carbohydrates(g/day) | 327.73±2.05 | 333.78±2.05 |
| Dietary fiber(g/day) | 13.46±1.75 | 32.63±1.75* |

[1] Values are means ± SEM
[2] Preintervention nutrient intakes of the subjects, obtained by two 24-h food recalls and food frequency
[3] Postintervention (after RSP supplementation) nutrient intake of the subjects
*Significantly different from baseline $p<0.05$

Table 2.5 summarizes the serum lipid concentrations of the participants at baseline, response after 4 weeks of RSP supplementation and washout effects after 4 weeks of RSP withdrawal. A significant decrease of 8% in total cholesterol concentration was observed after 4 weeks of fiber treatment as compared to subject's baseline values ($p<0.05$). The major reduction was observed in LDL - cholesterol fraction with depressions as large as 20% ($p<0.05$). When the RSP supplement was withdrawn, the depressed serum total and LDL-cholesterol values rose again and approached baseline in 4 weeks. Serum HDL-cholesterol concentrations were not influenced by either RSP supplementation or refrainment. A trend towards lowering the triglyceride concentration was also present.

**Table 2.5** ***Effect of oral administration and withdrawal of rhubarb stalk powder (27g/day) for 4 weeks on the serum lipid profiles of the hypercholesterolemic men[1,2]***

| | Preintervention[3] | Postintervention[4] | Withdrawal effect[5] |
|---|---|---|---|
| | | mmol/L | |
| Total-chol | 6.58 ± $0.18^a$ | 6.06 ± $0.18^b$ | 6.43 ± $0.18^a$ |
| LDL-Chol | 4.42 ± $0.18^a$ | 4.03 ± $0.18^b$ | 4.31 ± $0.18^a$ |
| HDL-Chol | 1.21 ± $0.02^a$ | 1.19 ± $0.02^a$ | 1.19 ± $0.02^a$ |
| TG | 2.12 ± $0.12^a$ | 1.80 ± $0.12^a$ | 1.83 ± $0.12^a$ |

[1] Values are means ± SEM
[2] In each column values not sharing a common superscript letter are significantly different at $p<0.05$.
[3] Baseline serum lipid concentrations, n=10
[4] Serum lipid concentrations after 4 weeks of RSP supplementation, n=10
[5] Serum lipid concentrations after 4 weeks of RSP withdrawal, n=8
Chol : Cholesterol; TG : Triglycerides; RSP : Rhubarb Stalk Powder

Figure 2.2 illustrates the individual responses of the subjects before and after the RSP supplementation. All the participants responded to RSP through decreases in serum total cholesterol concentrations and the reductions ranged from 3 to 17%. In 7 of the 8 subjects the depressed cholesterol levels returned to normal after 4 weeks of RSP withdrawal.

## *2.4 DISCUSSION*

The study provides evidence that the cholesterol lowering action of RSP in the hypercholesterolemic men is consistent with its effect in hypercholesterolemic animals (Basu et al., 1993). The new fiber source significantly lowered both serum total and LDL - cholesterol without affecting the HDL-cholesterol concentrations. The magnitude of reduction was higher than that reported with the ingestion of mixed fiber sources such as barley bran flour (Lupton et al., 1994) and rice bran (Kestin et al., 1990), and was similar to that obtained with soluble fibers such as guar gum (Superko et al., 1988) and psyllium (LaRosa, 1990). A trend of lowering the triglyceride concentrations was also present, but, the results were not statistically significant. It was of interest, however, that the reduced concentrations of triglycerides did not return to baseline values in 4 weeks of washout. Perhaps, much larger sample size was required to assess the true hypotriglyceridemic effects of RSP.

The dietary supplement of 27g of RSP was well tolerated by all the subjects and was not associated with any adverse effects. Two subjects initially reported mild bloating and loose feces but they all in fact felt improvements in their bowel movements with time

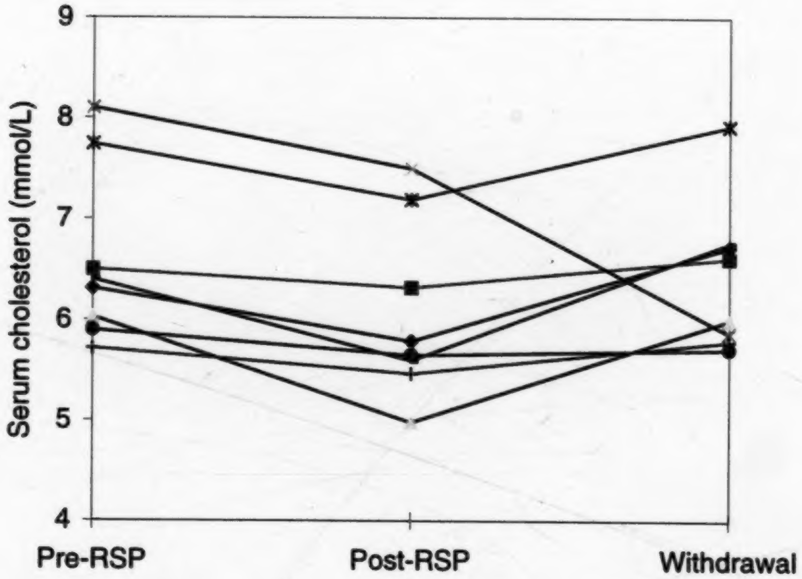

**Fig 2.2 Effect of dietary supplementation of RSP (27g/day) for 4 weeks on serum cholesterol concentrations of hypercholesterolemic men : Individual responses.** n=8

and some requested more fiber after the completion of the study.

The compositional data of RSP reveals that, though, the product contained appreciable amounts of protein, ash, oxalic acid and malic acid (Table 1.3), the major fraction was dietary fiber. Therefore we speculate, that perhaps, the high fiber content of RSP was responsible for the observed effects. However, because of the higher content of insoluble fiber, the role of fiber in these cholesterol lowering effects could be disputed, because these kind of fibers have generally been found to be less effective than soluble fibers as hypocholesterolemic agents (Jenkins et al., 1975; Kashtan et al., 1992; McIntosh et al., 1991). However, the relative ineffectiveness of insoluble fiber cannot be generalized to all sources, especially to those containing fractions of both soluble and insoluble fibers because some mixed fibers such as soy fiber and barley bran have also proved effective as hypocholesterolemic agents (Lupton et al., 1994; Kestin et al., 1990; Lo et al., 1986; Sasaki et al., 1985). RSP also has a fraction of soluble fiber (8%), mainly as pectin. Pectin, because of its gelatinous nature, high fermentability and ability to trap bile acids, has been shown to be an efficient hypocholesterolemic agent (Basu & Ooraikul, 1995). Therefore, it is also plausible, that the consistent cholesterol-lowering effects of RSP could be due to the portion of pectin. Additionally some insoluble fibers such as alfalfa (Kritchevsky et al., 1975; Eastwood & Hamilton, 1968) or insoluble fractions in mixed fibers such as lignin (Judd et al., 1975) complex with bile salts and hypocholesterolemic effects of alfalfa in hypercholesterolemic rabbits seem attributable to increased fecal excretion of steroids (Kritchevsky et al., 1974). High excretion of bile acids has been shown to stimulate hepatic LDL-receptor activity, consequently to increase the catabolic rate of LDL-cholesterol leading to reduction of plasma LDL-cholesterol concentrations (Shepherd et al., 1980). However, RSP in addition to dietary fibers, also contains some associated components such as proteins and ash. Some evidences have indicated the cholesterol depressing effects of soy protein (Potter et al., 1993) and ability of dietary calcium to bind bile salts in intestinal lumen (Lipkin & Newmark, 1995). Thus, the contributory effects of these components to cholesterol lowering effects of pectin fraction of RSP cannot be ignored. Some other insoluble fiber sources such as brewer's spent grain elicit cholesterol-lowering effects because of the presence of α-tocotrienol, a plant sterol possessing isoprenoid ring that has been reported to repress HMG-CoA reductase, the rate limiting enzyme for cholesterol synthesis (Newman et al., 1989). However, since RSP did not contain any lipids its unlikely that phytosterols could be involved in the observed effects. This study does not delineate the mechanism of action of RSP *in situ* and further studies are required to investigate its influence on absorption and fecal excretion of sterols, fats and bile acids.

Some investigators suggest that fiber may exert its cholesterol lowering effect by displacing other fat foods in the diet (Swain et al., 1990). However, the changes in the lipid concentrations observed in our study could not be attributed to changes in the diet since no significant changes in any nutrient intake were observed during the 4 week clinical trial except for dietary fiber. The return of depressed cholesterol levels to baseline values after 4 weeks of RSP withdrawal further strengthen the role of this fiber source in the observed hypocholesterolemic effects.

The results of the present study suggest that RSP is a valuable source of dietary fiber for the management of hypercholesterolemia in patients with undesirably high serum cholesterol levels. An additional benefit of RSP is that it is chiefly an insoluble fiber source. In general, insoluble fibers accelerate colonic transit and increase fecal bulk, two factors thought to be protective against colon cancer.

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# CHAPTER 3

## *In vitro binding of bile salts to rhubarb stalk powder*[1]

### *3.1 INTRODUCTION*

Dietary fiber consists of a diverse group of substances that can vary widely in chemical and morphological properties (Roberfroid, 1993). Soluble fibers are generally considered as better hypocholesterolemic agents than insoluble types of fibers (Jenkins et al., 1975 ; Anderson et al. 1992). Viscosity (Gallaher et al. 1993) and fermentability ( Anderson & Chen, 1979) are considered to be the major attributes of soluble fibers responsible for these effects. However the physiological relevance of these properties is still disputed, because some feeding trials using highly viscous fibers, such as methyl cellulose, could not produce any effects on plasma cholesterol concentrations (Topping et al.1988). Additionally, although oral propionate has been shown to inhibit endogenous cholesterol synthesis, the concentrations required are several fold higher than those found *in vivo* (Illman et al., 1988). Except for fibers, such as barley and oat fibers (Newman et al. 1989 ; Drennan, 1991), which contain phytosterols (α-tocopherol and saponins), fibers that have proved effective in lowering plasma cholesterol concentrations usually affect bile acid metabolism.

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[1] A version of this paper has been submitted to *British Journal of Nutrition*

In this regard most of these fibers have shown acceleration in fecal bile acid losses (Garciadiez et al. 1996) consequently, changing biliary bile acid composition (Matheson & Story, 1994) and upregulating cholesterol 7α-hydroxylase activity (Horton et al.1994, Matheson et al., 1995). Some of these fibers, both soluble and insoluble complex with bile salts *in vitro*, and seem to mimic ion exchange resins *in vivo* (Gallaher & Schneeman, 1986).

Both experimental and clinical trials have indicated that RSP is a valuable source of dietary fiber for the management of hypercholesterolemia (Basu et. al., 1993, Chapter-2). The underlying mechanism by which these effects are achieved is unknown. Since RSP contains a small fraction of soluble fiber, it is unlikely that viscosity could be the major attribute responsible for the hypocholesterolemic effects. To investigate a probable mechanism of action, the fermentability and the ability of RSP to form complexes with bile salts, such as taurocholate or cholic acid, was determined *in vitro* and compared to that of other commonly used fiber sources.

## *3.2 MATERIALS AND METHODS*

### *3.2.1 RSP*

Fresh rhubarb stalks were obtained from growers and food stores in Edmonton. The stalks were cut into 3 cm length and steam cooked for 10 minutes before squeezing them with a manual screw press to remove the juice. The pressed stalks were leached twice in about 10 times their weight of water at 70°C for 15 minutes and squeezed before being dried in a fluidized bed dryer at 85°C for 1h 40 minutes. Subsequently, the dry stalks were ground finely. The ground fiber was sifted through a 16 mesh sieve and was analyzed for chemical composition (Table 3.1).

**Table 3.1** ***Chemical composition of RSP***[1,2]

| | %, Dry weight |
|---|---|
| Protein | 5.6 |
| Ash | 5.6 |
| Oxalic acid | 5.7 |
| Malic acid | 3.3 |
| Insoluble dietary fiber | 65.9 |
| Soluble dietary fiber | 8.2 |
| Total dietary fiber | 74.1 |

[1] Values are means of 2-4 replicates.

[2] Insoluble and soluble dietary fiber contents were estimated by the method of Prosky et.al (1988). Oxalic acid and malic acid were determined by the procedure of Kok et al.(1984).

### 3.2.2 *Fermentation*

The fiber sources were fermented *in vitro* with mixed human fecal microbiota using the method of McBurney and Thompson (1987). Fermentations were repeated with three donors to more accurately estimate *in vivo* colonic SCFA production. Briefly, 0.5g samples were weighed into 100-ml serum bottles. Fermentation medium (40 ml) containing 2.49 g/L trypticase peptone, 1.00 g/L ammonium biocarbonate, 8.75 g/L sodium bicarbonate, 1.43 g/L anhydrous sodium phosphate, 1.55 g/L anhydrous potassium phosphate monobasic, 0.60 g/L magnesium sulfate, 0.12 mg/L resazurin, 1.12 mM calcium chloride, 0.63 mM manganous chloride, 0.15 mM cobalt chloride, and 0.04 mM ferric chloride was added to the samples for 12 to 24 h before the start of the incubation so that the samples would be hydrated when the inoculum was added. The contents of the serum bottles were reduced, and the bottles were sealed with a butyl rubber stopper and crimped metal seal and stored overnight in the refrigerator. One to 2 h before inoculation, the bottles were placed in a 37°C water bath.

Fresh human feces were collected from three healthy volunteers who had not taken antibiotics for atleast 3 mo and had each supplemented their regular diets for 5 days with 7-14 g dietary fiber as Fibrad. Each fecal sample was collected into a tared blender containing a known volume of collection medium that had been warmed to 37°C and was oxygen free. The collection medium consisted of distilled water, fermentation medium, and reducing solution (15:15:2 vol/vol/vol). The feces were diluted with the collection medium (66.6 g wet feces/L) blended for 30 s and squeezed through a 41 μm nylon membrane to remove fibrous particles. Ten milliliters of this inoculum was injected into each serum bottle. Serum bottles were swirled at regular intervals. The fermentation was terminated at 24 h by opening the serum bottles and adding 1 ml copper sulfate (20 g/L) as a bacteriocide.

A 1.5 ml aliquot was removed for Short Chain Fatty Acid (SCFA) analysis by HPLC. Briefly, the 1.5 ml aliquot was centrifuged at 10,000 rpm to sediment microbial and particulate matter. The supernatant (30 μl) was directly injected onto a BioRad HPX-87H organic acid column kept at 50°C, and absorbance was measured at 214 nm. The eluant was 0.005 N $H_2SO_4$ at a flow rate of 0.6 ml/min. Individual and total SCFAs were expressed as per gram organic matter weighed into the serum bottle.

### *3.2.3 Binding studies*

#### *3.2.3 1 Experiment 1*

The assay for *in vitro* binding of bile salt was done using a method described previously (Hangerman et al. 1973). Briefly, 200 mg samples of various fibers and colestipol (Colestid, Upjohn Pharmaceuticals) were incubated with 10 mM [24-$^{14}$C] taurocholic acid (specific activity of 2000 dpm/μmol) in 5 ml phosphate buffer (pH 7.4) at 37$^0$C for 2 h. Following the incubation, a 3 ml mixture was centrifuged at 30,000xg for 10 minutes. A 0.5 ml aliquot was counted for radioactivity in liquid scintillation counter. The amount of bile salt bound was calculated as the difference between the amount of bile salt added and that recovered in the supernatant.

#### *3.2.3.2 Experiment 2*

The concentration dependent effect of RSP on bile salt binding was also investigated through a separate experiment. The various amounts of rhubarb stalk powder or cellulose (100 - 300 mg) were incubated with 10mM of labeled taurocholate and the amount of bile salt bound was calculated as described above.

#### *3.2.3.3 Experiment 3*

The effect of bile salt concentration on the ability of RSP to bind bile salts was also determined. RSP or cellulose (200 mg) were incubated with 2 to 10 mM of labeled taurocholate and the amount of bile salt bound was calculated as stated above.

#### *3.2.3.4 Experiment 4*

To investigate if the conjugation of bile salt was essential for binding to RSP, solutions containing 10 mM labeled taurocholate with a specific activity of 2000 dpm/μmol and increasing concentrations of cholic acid (10 mM to 20 mM) were prepared. 200 mg RSP or cellulose was incubated in 5 ml of these solutions and the ability of cholic acid to compete/inhibit the binding of taurocholate to fibers was calculated as described earlier.

## *3.3 RESULTS*

Table 3.2 elicits the results of *in vitro* fermentation of various fibers using human fecal bacteria after 24 h. The SCFA production was maximum from the fermentation of pectin and minimum from cellulose. Soy and rhubarb fibers showed the moderate fermentability in between that of highly fermentable pectin and less fermentable cellulose fiber. The ratio of acetate to propionate in RSP (5.13) was similar to pectin (5.30) and they showed higher ratio as compared to soy or cellulose fibers (2.78 or 2.29).

**Table 3.2** ***24-h SCFA production from fermentation of fibers using human fecal bacteria*[1,2]**

| SCFA (mmol/g) | RSP | Soy fiber | Cellulose (alphacel) | Pectin |
|---|---|---|---|---|
| Acetic | 4.88 | 4.47 | 1.08 | 7.64 |
| Propionic | 0.95 | 1.61 | 0.47 | 1.44 |
| Isobutyric | 0.12 | 0.19 | 0.14 | 0.24 |
| Butyric | 0.74 | 1.04 | 0.37 | 1.40 |
| Iso-Valeric | 0.09 | 0.16 | 0.25 | 0.33 |
| Valeric | 0.18 | 0.24 | 0.16 | 0.34 |
| Acetate/propionate | 5.13 | 2.78 | 2.29 | 5.30 |
| Total | 6.94 | 7.71 | 2.47 | 11.39 |

[1] Values are means of three independent determinations

Table 3.3 summarizes the bile salt binding capacity of RSP relative to colestipol, an anion exchange resin and bile acid sequestrant, and several commonly used fiber sources such as wheat bran, corn bran, rice bran and cellulose. Colestipol bound more taurocholate than any of the fibers tested. Among the fibers tested RSP exhibited the highest capacity for binding. It bound 2 to 3 fold as much bile salt than corn bran, rice bran or wheat bran, and 11 fold more bile salt than cellulose.

**Table 3.3** ***Binding of Taurocholate by various fibers (In vitro)***[1,2]

| Fiber | Taurocholate bound (μmol/200mg) |
|---|---|
| Colestipol | 41.77 ± 0.10[a] |
| Cellulose | 1.89 ± 0.87[b] |
| Corn Bran | 6.86 ± 0.68[c] |
| Rice Bran | 6.93 ± 0.65[c] |
| Wheat Bran | 4.52 ± 0.96[c] |
| Rhubarb Stalk Fiber | 12.34 ± 1.09[d] |

[1] Values are means ± SEM of 5 independent determinations.
[2] Values not sharing a common superscript letter are significantly different at $p<0.05$.

The effect of increasing RSP concentration on binding to taurocholate was also evaluated. As the concentration of RSP increased, the binding increased linearly in concentration dependent fashion (Fig 3 1). In contrast, cellulose did not show any appreciable increase in binding even at the highest concentration.

Addition of increasing amounts of taurocholate to a fixed amount of RSP showed a linear increase in binding up to a concentration of 10 mM (Fig 3.2). No further increase in binding was observed beyond this concentration. Thus, the RSP has a binding capacity of 40 µmol per gram. Cellulose, exhibited a small increase in binding with increasing concentrations of taurocholate but to a much lesser extent than RSP.

We also investigated if the conjugation of bile salt was essential for the binding. As shown in Fig 3.3, cholate competed effectively taurocholate for binding. At equimolar concentrations of both conjugated taurocholate and unconjugated cholic acid, the amount of taurocholate bound was reduced by 50%. Thus, conjugation of bile salt was not a prerequisite for binding to RSP.

## *3.4 DISCUSSION*

Degradation of cholesterol to bile acids is one of the major pathways by which cholesterol is eliminated from the body. Bile acid sequestrants such as cholestyramine and colestipol have for many years been considered to be a useful drug therapy for patients with hypercholesterolemia ( Ast & Frishman, 1990). The sequestrants work by binding the bile salts in the gut lumen and thus inhibit their reabsorption and facilitate their excretion. The bile salt binding ability of these agents has been demonstrated *in vitro* ( Zhu et al. 1992) as well as *in vivo* ( Gaw et al. 1996). However, because of the undesirable side effects associated with their continuous use (Ast & Frishman, 1990) and their low *in vivo* potency (Benson et al. 1993), dietary modifications such as lowering the intake of fat and increasing the intake of dietary fiber have emerged as the first choice of therapy to manage mild to moderate hypercholesterolemia (Goodman, 1988).

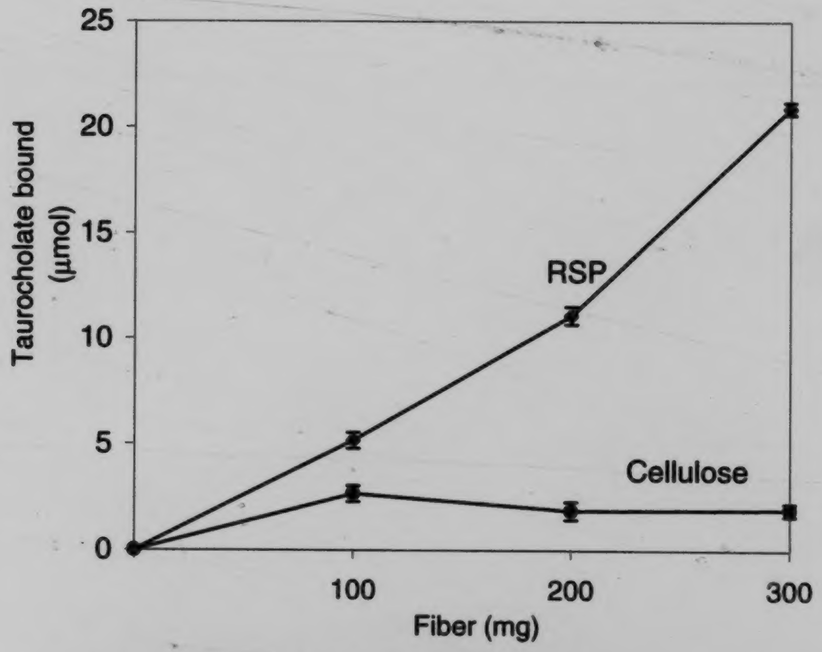

**Fig 3.1 Effect of RSP or cellulose concentration on its binding to taurocholate.** All values are means ± SEM of 5 independent determinations and are significantly different at $p<0.05$.

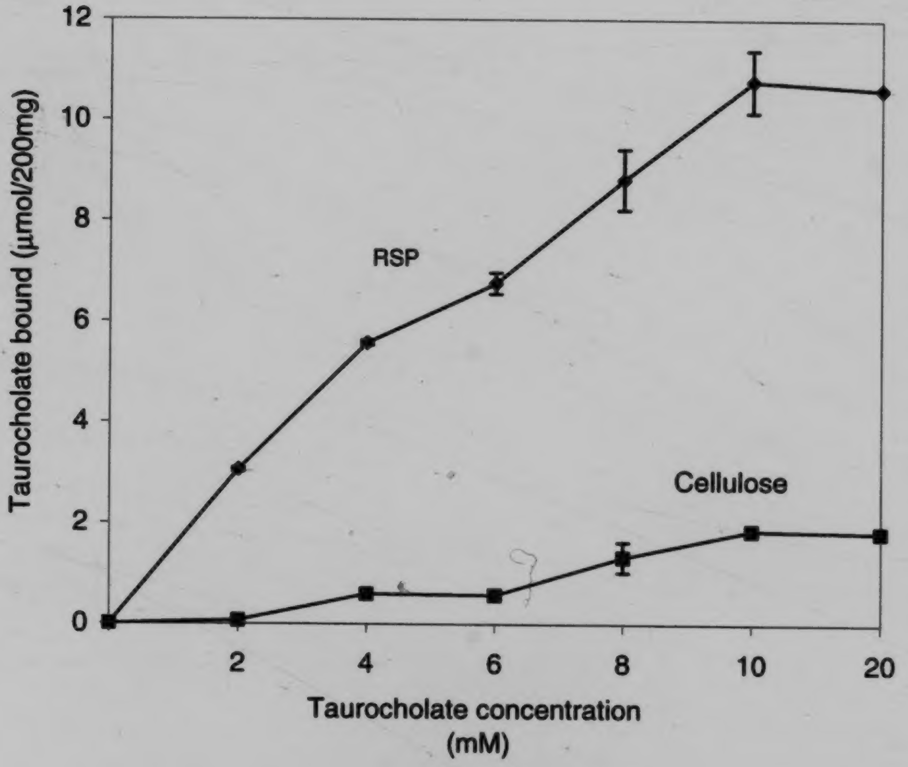

**Fig 3.2 Effect of taurocholate concentration on its binding to RSP or cellulose.** All values are means ±SEM of 4 independent determinations and are significantly different at $p<0.05$.

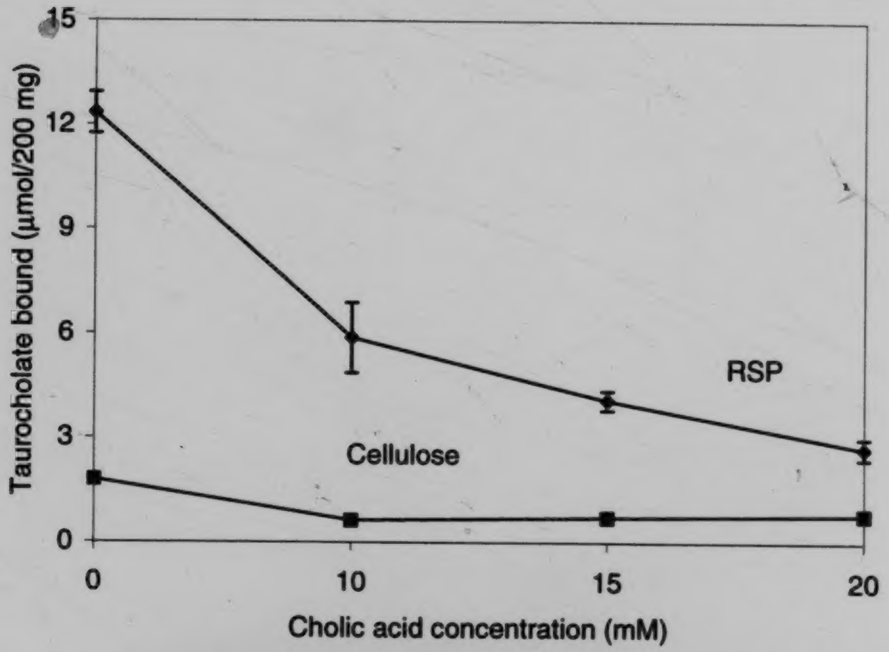

**Fig 3.3 Competitive binding between cholic acid and taurocholate to RSP or cellulose.** All values are means±SEM of 4 independent determinations and are significantly different at $p<0.05$.

Many fibers, especially the soluble fibers such as psyllium (LaRosa, 1990) and pectin (Tinker et al. 1991) have proved effective in this regard. The properties of fibers that have been related with their ability to reduce cholesterol include viscosity, fermentability and the ability to entrap bile salts. Although some studies have indicated that bile acid binding capacity of non-viscous and nonfermentable fibers was not sufficient to lower plasma cholesterol concentrations (Zacour et al., 1992), it does appear to be a pre-requisite to achieve significant lowering of plasma cholesterol levels with dietary fibers (Moundras et al., 1994). Other properties such as fermentability and viscosity are also desired. It is noteworthy, that RSP despite having most of the fiber as insoluble elicited significant cholesterol lowering effects in animals as well as in humans. The novel ability of RSP to bind bile salts and its moderate fermentability as illustrated in this study could be responsible for its repeated lipid lowering effects. Compared to the other fibers tested here, RSP is second only to colestipol in ability to bind taurocholate. This binding capacity is significant considering that, unlike colestipol the natural source was not specifically modified for this purpose. It was noteworthy that although fiber sources such as corn bran (Hunningshake et al. 1994) and rice bran ( Kestin et al. 1990) are also mixed fibers and have exhibited cholesterol lowering effects in experimental and clinical trials, their potency to bind taurocholate was much lower than RSP. The relative ineffectiveness of wheat bran and cellulose fibers in binding with bile salts is consistent with the previous observations (Story & Kritchevsky, 1976) and these fibers are also not associated with any cholesterol lowering effects (Jenkins et al. 1975 ; Kashtan et al. 1992).

The specific component in RSP responsible for bile salt binding, although, is not known, this appears to be an effect of unique combination of factors present in the fiber source. The product contains predominantly insoluble type of fibers. Although cellulose, the most abundant insoluble polysaccharide, did not show any appreciable binding, other insoluble noncellulosic polysaccharides and nonpolysaccharides such as hemicelluloses (Norman et al. 1987) and lignin ( Gallaher & Schneeman, 1986) have been shown to possess appreciable adsorption capacity for bile salts. Additionally, RSP also exhibits high water holding capacity and absorbs up to 19 times its weight of water (Unpublished observation). Thus, it is plausible that the rhubarb fiber's high hydration capacity and content of soluble fiber (8%) could have lead to non-specific entrapment of bile salt in the interstitial space of the fiber matrix or in the gelatinous mass formed by the soluble portion of the dietary fiber. Highly viscous fibers such as guar gum (Ebihara & Schneeman, 1989) have also been shown to adsorb bile salts and to increase fecal bile salt excretion (Garciadiez et al. 1996). RSP also contains appreciable amounts of minerals particularly calcium (Goel et al., 1996). Calcium has been shown to have the ability to sequester bile salts (Govers et al. 1994). Therefore, it is conceivable that combination of fibers, both soluble and insoluble, and other associated components such as minerals in RSP might have granted it a novel ability to complex with bile salts and therefore elicit cholesterol-lowering effects.

*In vitro* studies have revealed that the bile salt structure is also an important determinant of bile salt affinity to the sequestrants (Hangerman et al. 1973). Sorbents such as cholestyramine possess greater affinity for taurine conjugated bile salts because of the ionic interaction between the ammonium groups of resin and acidic sulfonic groups of taurine (Zhu et al. 1992). However, our competitive binding assay between conjugated taurocholate and unconjugated cholic acid, revealed that RSP had an equal affinity for both conjugated and unconjugated bile salts. Thus, suggesting that there is limited involvement of taurine in binding of RSP to bile salts.

The moderate fermentability exhibited by RSP might be related with its fractions of both soluble and insoluble dietary fibers, since pectin (soluble fiber) showed the highest fermentability and cellulose (insoluble fiber) had the lowest fermentation. Although the total production of SCFA from RSP fermentation resembled that from soy fiber, the molar percentage of propionic acid was higher with soy fiber than with RSP. Propionic acid has been suggested to inhibit HMG-CoA reductase, thus inhibiting cholesterol synthesis. Some evidence also indicate that the ratio of acetate to propionate produced during fermentation of fibers determine its cholesterol lowering effects, with a positive relationship between serum acetate to propionate ratio and total and LDL-cholesterol (Wolever et al., 1996). Despite showing lower molar concentration of propionic acid than soy fiber or pectin, the ratio of acetate to propionate produced from RSP fermentation was similar to that from pectin, thus its likely that moderate fermentability of RSP could have also contributed to its ability to lower plasma cholesterol concentrations.

In conclusion, this study highlights an important property of RSP, to bind with bile salts. This property could be responsible for its consistent hypocholesterolemic effects in experimental and clinical trials.

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# CHAPTER-4

## Dietary rhubarb stalk powder (RSP) stimulates cholesterol 7α-hydroxylase gene expression and bile acid excretion in cholesterol fed C57BL/6J mice[1]

### *4.1 INTRODUCTION*

Dietary fiber has become increasingly a treatment of choice to combat mild to moderate hypercholesterolemia. Fibers with predominantly water-soluble components have proved to be more effective than insoluble types of fibers as hypocholesterolemic agents (Jenkins et al. 1975 ; Anderson et al. 1992). Multiple mechanisms have been suggested in cholesterol lowering effects of these fibers including disruption of micelle formation due to viscosity thus leading to lipid malabsorption (Gallaher et al. 1993), suppression of hepatic sterol synthesis by fermentation products of dietary fiber (Kishimoto et al. 1995), and binding or sequestration of bile acids causing the interference of the enterohepatic recirculation of bile acids and an increase in fecal bile acid excretion (Fernandez,1995 ; Matheson et al. 1995).

Recently, a predominantly insoluble fiber source, rhubarb stalk fiber (RSP), has been found to depress the plasma total and LDL-cholesterol concentrations in experimental animals fed high cholesterol diets (Basu et al.1993) and in clinical trials involving hypercholesterolemic subjects (Chapter 3).

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[1] A version of this paper has been submitted to *British Journal of Nutrition*

The precise mechanism by which this effect is achieved still remains unknown. The evidence to date suggest that the fiber does not elicit hypocholesterolemic effects through inhibition of the hepatic cholesterol synthesis (Basu et al. 1993).Additionally, since RSP is an insoluble fiber source having less fermentability than soluble fibers, it is unlikely that its cholesterol lowering effects is mediated predominantly through interference with the absorption of lipids or micelle formation due to viscosity, or via the fermentation products such as short chain fatty acids. However, recent *in vitro* experiments have shown that RSP has a capacity to form complexes with bile salts (Chapter 4).

Degradation of cholesterol to bile acids is the major pathway by which cholesterol is eliminated from the body and hepatic cholesterol 7α-hydroxylase is the rate limiting enzyme in this process (Russel & Setchell, 1992). Although the precise mechanisms involved in the regulation of cholesterol 7α-hydroxylase activity and gene expression are not fully understood, several studies have demonstrated that the enzyme activity and mRNA abundance are induced by dietary cholesterol and the enzyme is subject to feed back inhibition by bile acids returning to the liver via enterohepatic circulation (Russel & Setchell, 1992). Thus, decreased absorption of bile acids due to strong binding by cholestyramine increases hepatic cholesterol 7α-hydroxylase activity and mRNA by 3-4 fold (Horton et al. 1994). It is, therefore, likely that RSP by virtue of its ability to bind with bile salts might be eliciting hypocholesterolemic effects via increasing fecal bile salt excretion.

The present study was undertaken to determine the effects of RSP on fecal and biliary concentration of bile acids in mice fed diets with or without dietary cholesterol and to investigate if these changes correlated with the changes in the expression and activity of cholesterol 7α-hydroxylase. C57BL/6J mice were used as experimental animals because of the increased sensitivity of this strain of mice to diet induced hypercholesterolemia (Paigen et al., 1987) and male animals were selected because of the greater responsiveness of males to dietary fiber in comparison to females (Fernandez et al., 1995).

## *4.2 MATERIALS AND METHODS*

### *4.2.1 Materials.*

[$^{14}$C] Palmitoyl CoA (55 mCi/mmol) was obtained from American radiolabelled chemicals and $^{32}$P-UTP was purchased from New England Nuclear, Boston, MA. Cholesterol oxidase was obtained from Boehringer Manheim Canada LTD (cat # RC 393924).

### *4.2.2 Animals and diets.*

Male, C57BL/6J mice (obtained from Jackson laboratory, Canada), 8 weeks old, weighing 16-20g were used. Mice were housed (2 per cage) in hanging stainless steel cages in a well ventilated room maintained at 21 ± 2°C and were on 12-h light-dark cycle. All animals were fed a pelleted diet (Purina Lab Rodent diet #5001, Purina, Richmond, IN) for one week before being fed an experimental semi-synthetic diet (Table 4.1). The animal protocol of the study was approved by the University of Alberta Animal Welfare Committee.

Mice were randomly divided into 4 groups of 6 animals each. The groups received diets containing either 5% cellulose or 5% RSP with or without 0.5% cholesterol added at the expense of corn starch. All diets contained olive oil (10%) as a source of dietary fat to exacerbate the hypercholesterolemic potential of dietary cholesterol (Cheema et al. 1997).

**Table 4.1.** ***Composition of the semi - synthetic diets****

| | Diets | | | |
|---|---|---|---|---|
| Ingredients | RSP | Cellu | RSP+Chol | Cellu+Chol |
| | | (%) | | |
| Casein | 20 | 20 | 20 | 20 |
| Corn Starch | 59.5 | 59.5 | 59.0 | 59.0 |
| Olive oil | 10 | 10 | 10 | 10 |
| Cholesterol | 0 | 0 | 0.5 | 0.5 |
| Vitamin Mix† | 2 | 2 | 2 | 2 |
| Mineral mix†† | 3.5 | 3.5 | 3.5 | 3.5 |
| Cellu | 0 | 5 | 0 | 5 |
| RSP | 5 | 0 | 5 | 0 |

*Ingredients were from ICN Biochemicals, Cleveland, Ohio, USA.

† AIN Vitamin mix (mg/ Kg diet): retinyl acetate 19.8; ergocalciferol 1.38; dl-α-tocopheryl acetate 110; ascorbic acid 495; inositol 55; choline 2227; menadione 24.7; p-aminobenzoic acid 55; niacin 46.7; riboflavin 11; pyridoxin HCl 11; thiamin HCl 11; D-Calcium pantothenate 33; biotin 0.2; folic acid 0.99; vitamin B12 0.015.

†† AIN Mineral mix (g/Kg diet): calcium phosphate dibasic 15; sodium chloride 2.2; potassium citrate monohydrate 6.6; potassium sulfate 1.56; magnesium oxide 0.7; manganous carbonate 0.105; ferric citrate 0.18; zinc carbonate 0.048; cupric carbonate 0.009; potassium iodate 0.0003, chromium potassium sulfate 0.0165.

RSP: Rhubarb Stalk Powder; Cellu: Cellulose ; Chol: Cholesterol.

### *4.2.3 Sample collection.*

Body weight and daily food intake of all the animals were recorded once a week. Two 24-h fecal samples were collected from each animal towards the end of the feeding period and were stored at -40$^0$ C until analysis. After 4 weeks of dietary treatment, the animals were fasted overnight and anaesthetized with halothane vapor for sample collection. Blood was drawn by cardiac puncture into plastic tubes containing anticoagulant (2.2 mmol EDTA dipotassium salt/L blood) and centrifuged at 1200xg for 20 min. at 4°C to obtain plasma. Livers were excised, blotted, weighed and quickly frozen in liquid nitrogen. Gall bladder bile was obtained by aspiration using a syringe with 25-gauge needle. Separated plasma, gall bladder bile and livers were stored at -80°C until analysis.

### *4.2.4 Analysis of plasma lipids.*

Plasma total cholesterol, HDL-cholesterol and triglycerides were determined in duplicate by enzymatic kits obtained from Sigma Biochemical (Catalog # 352-3, 352-20 and 336-10 respectively). LDL-cholesterol was calculated as described by Friedwald et al (1972). Free cholesterol levels in plasma were estimated by enzymatic kit obtained from Boehringer Manheim Canada Ltd. (catalog # 139050 ) lacking esterase. Cholesterol esters were calculated as the difference between the total and the free cholesterol.

### *4.2.5 Analysis of Liver lipids.*

The total lipids from liver samples were extracted using the chloroform - methanol (2:1 v/v) extraction procedure of Folch et at.(1957). The concentrations of total cholesterol, triglycerides and free cholesterol were determined by above stated enzymatic methods.

### *4.2.6 Analysis of fecal lipids.*

Fecal samples were freeze dried, ground and analyzed for total cholesterol and total bile acids using ethanol and petroleum ether extraction (Malchow -Moller et al. 1982). Briefly, 200 mg fecal material was vortexed with 2 ml of acidified ethanol (10 ml of 50% acetic acid in 90 ml of ethanol), centrifuged at 2000 rpm for 5 min. Supernatant obtained after centrifugation was dried under nitrogen and redissolved in 2 ml of 0.1 M NaOH. Cholesterol was then extracted twice with 2 ml of petroleum ether. The pooled petroleum ether was dried under nitrogen, resuspended in 2 ml of isopropanol and 10 µl of this was used for the estimation of cholesterol using Sigma enzymatic kit. The bottom layer obtained after the addition of NaOH was acidified with 2 ml of 1 M HCl and the bile acids were then extracted twice with 2 ml of ethyl acetate. The pooled ethyl acetate was evaporated to dryness under nitrogen, resuspended in 1 ml of methanol and 20 µl was used for the determination of total bile by 3α-steroid dehydrogenase method (Sigma Diagnostic Canada, Catalog # 450-A).

### *4.2.7 Analysis of biliary lipids.*

Total cholesterol concentration in the bile was determined by diluting 1 μl of bile to 20 μl with distilled water and then estimation of cholesterol in 10 μl of the sample by enzymatic kits as described earlier. For the quantification of bile acids in the bile 2μl of bile was diluted to 200 μl with distilled water and the bile acids were then estimated using enzymatic kit from Sigma Biochemicals.

### *4.2.8 Cholesterol 7α-hydroxylase assay*

Samples of frozen liver were used for the preparation of microsomes (Garg et al., 1989). Briefly, 200-300 mg tissue was homogenized in ice cold buffer containing 0.3M sucrose, 1mM EDTA, 50mM KCl, 0.1M $K_2HPO_4$ (pH 7.4) with five up and down strokes of a Potter - Elvehjem tissue homogeniser. Homogenates were centrifuged at 10,000 rpm at 4°C for 20 minutes to remove the cell debris. The supernatant was recentrifuged for 70 min. at 35,000 rpm in a SW-60 rotor (Beckman instruments) at 4°C. The microsomal pellet obtained from the second spin was resuspended in 500μL of buffer containing 0.1M $K_2HPO_4$ (pH 7.4), 1mM EDTA, 50mM KF, 5mM DTT and 50mM KCl. Aliquots of liver microsomes were used for protein estimation (Lowry et al., 1951) and then quickly frozen in liquid nitrogen and stored at -80°C until analysis.

The cholesterol 7α-hydroxylase activity was measured using a modification of a previously described method of Chiang,1991. Briefly, 1mg microsomal protein was incubated in 1ml of buffer containing 0.1M $K_2HPO_4$ (pH 7.4), 1mM EDTA, 50mM KF, 5mM DTT and 0.015% CHAPS. The mixture was preincubated for 5 min. at 37°C and the reaction was initiated by addition of 100 μL of 10 mM NADPH. The samples were incubated for 30 min. at 37°C and the reaction was terminated by the addition of 30 μL of 20% sodium cholate. Cholesterol oxidase (45 U/mg) was then added to oxidize the products of the reaction. After 15 min., the assay mixtures were extracted twice with 4 ml of petroleum ether, dried and stored under nitrogen until analysis.

The residue was resuspended in 100 μL of acetonitrile/methanol (70:30) and a 20 μL aliquot was analyzed by HPLC (Beckmen System Gold) on a 4.6 x 250 mm Ultrasphere column (Beckmen) with running solvent (acetonitrile/methanol, 70:30) at a flow rate of 0.8 ml/min. The absorbance of the sample was measured at 240 nm. The 20α-, 7α- and 7β-hydroxy-4-cholesten-3-one derivatives eluted at about 8, 12 and 15 min., respectively.

### *4.2.9 Acyl CoA : cholesterol acyltransferase assay (ACAT)*

ACAT was assayed by the method of Spector et al.1980. Briefly, 200 μg of microsomal protein was preincubated in 500 μL of buffer containing 0.1M $K_2HPO_4$ (pH 7.2) and 1 mM DTT for 5 min. at 37°C. The reaction was started by the addition of 10 nmol of [1-$^{14}$C]palmitoyl CoA (0.05 μCi). Incubations were carried out for 5 min. at 37°C with constant shaking and the reaction was terminated with the addition of 2 ml of chloroform / methanol (2:1). Lipids were extracted in the chloroform phase by the extraction procedure of Folch et al. 1957. Cholesterol esters produced as a result of ACAT action were separated by thin layer chromatography on silica gel-G plates, using a solvent system of light petroleum, diethyl ether and acetic acid (80:20:1 v/v). The bands across the cholesterol esters were scraped and counted directly in liquid scintillation counter. From the known specific activity of [1-$^{14}$C]palmitoyl CoA substrate, ACAT activity was expressed as pmol of cholesterol palmitate formed / min. / mg microsomal protein.

### *4.2.10 Determination of cholesterol 7α-hydroxylase mRNA abundance*

#### *4.2.10.1 Extraction of total RNA*

Total RNA from mouse livers was purified according to standard procedures (Chomczynski & Sacchi,1987). Briefly, 300 mg tissue was homogenized in a solution containing 4 M guanidium thiocyanate and 2 M sodium acetate with a few strokes in a glass teflon homogenizer. The homogenate was transferred into a 5-ml polypropylene tube and mixed sequentially with 0.1 ml of 2 M sodium acetate (pH 4), 1 ml of water saturated phenol, and 0.2 ml chloroform. The resulting mixture was centrifuged at 9000 rpm at 4$^0$C. The upper aqueous phase that contained the total RNA was transferred to a fresh tube. The RNA was then precipitated by adding 1 ml of 100% isopropanol and by freezing the samples at -70$^0$C for 30 min. The RNA pellet was obtained by centrifuging the frozen samples at 9000 rpm for 10 minutes at 4$^0$C. The pellet was redissolved in 0.3 ml solution of 4 M guanidium thiocyanate and again reprecipitated with 100% isopropanol. Finally, the pellet was washed with 75% ethanol and dissolved in 100 μl of DEPC (diethyl pyrocarbonate) - treated water. The RNA was quantitated by ultraviolet spectrophotometry at 280 nm and the purity of the RNA was determined by checking the integrity of 18s and 28s bands by running through formaldehyde agarose gel.

#### *4.2.10.2 Ribonuclease protection assay*

Hepatic cholesterol 7α-hydroxylase mRNA levels were determined by a ribonuclease protection assay (Cheema et al., 1997). Total RNA (20μg) was hybridized with $^{32}$P-labeled antisense probes for mouse 7α-hydroxylase (71 nt from intron 2 and 228 nt from exon 3 of the mouse 7α-hydroxylase gene) and mouse glyceraldehyde -3-phosphate dehydrogenase (G3PDH) synthesized from pTRI-G3PDH (Ambion), at 55°C overnight. Unhybridized probes were removed by treatment with RNAse one (Promega Biotech) (4U/μg RNA) for 1 h at 25°C. The protected mRNA fragments were separated on 5% polyacrylamide sequencing gels. The radioactivity in each band was quantitated by phosphorautoradiography using Fuji-X BAS 1000 plate imager. The amount of 7α-hydroxylase was normalized to G3PDH mRNA content.

### *4.2.11 Statistical analysis.*

The data was analyzed using SAS version 11.0. A two-way ANOVA was employed with dietary cholesterol and dietary fiber as main effects. ANOVA was followed by student's t-test to compare treatment means. Differences were judged to be statistically significant if the associated P value was <0.05 (Steel & Torie, 1987).

## *4.3 RESULTS*

Table 4.2 shows that the mice fed 5% RSP or cellulose fiber diets with or without 0.5% cholesterol remained unaffected in terms of their food intake, body weight gain, liver weights or fecal weights.

**Table 4.2** ***Effect of dietary RSP (5%) for 4 weeks with or without supplemental cholesterol (0.5%) on food intake, weight gain and fecal weights of mice[1,2].***

| Diets | Food intake (g/day) | Body weight gain (g) | Liver weight (g) | Fecal weight (g/24-h) |
|---|---|---|---|---|
| Cellu | 3.58 ± 0.32 | 4.05 ± 0.34 | 0.98 ± 0.03 | 0.28 ± 0.04 |
| RSP | 3.95 ± 0.20 | 3.91 ± 0.38 | 1.11 ± 0.04 | 0.28 ± 0.02 |
| Cellu+Chol | 3.81 ± 0.21 | 3.89 ± 0.51 | 1.15 ± 0.07 | 0.28 ± 0.03 |
| RSP+Chol | 3.78 ± 0.29 | 4.38 ± 0.54 | 1.14 ± 0.06 | 0.28 ± 0.04 |

[1] Values are means ± SEM of six animals.
[2] Differences among diets were non-significant at $p<0.05$.
RSP: Rhubarb Stalk Powder ; Cellu: Cellulose ; Chol: Cholesterol.

### *4.3.1 Effect of RSP on plasma lipids*

Enrichment of the diets with 0.5% cholesterol did not cause any appreciable increase in plasma cholesterol and triglyceride concentrations of the animals (Table 4.3). However, the hypercholesterolemia was evident through significant increase in plasma LDL-cholesterol and concomitant reduction in HDL-cholesterol concentrations. It was of interest that the group receiving RSP (5%) with 0.5% cholesterol had significantly ($p<0.05$) lower concentrations of plasma total cholesterol and cholesteryl esters as compared to the groups receiving a diet containing cellulose fiber (5%) with or without (0.5%) cholesterol supplement. The hypocholesterolemic effect was specific for LDL fraction. Mice fed cholesterol-enriched diets along with RSP had LDL - cholesterol levels similar to the animals maintained on cholesterol unsupplemented diets.

### *4.3.2 Effect of RSP on liver lipids*

Hypercholesterolemia in cholesterol fed animals was more pronounced in the liver than in the plasma (Table 4.4). It was, however, noteworthy that in RSP fed animals the increase in hepatic concentration of cholesterol was 1.5 fold less as compared to cellulose fed animals The major increase was observed in the cholesteryl-esters. Hepatic cholesteryl esters increased 8 fold in cellulose group as compared to 6 fold increase in RSP fed animals due to cholesterol supplementation. In parallel with the cholesteryl-ester concentrations, an upregulation of acyl CoA : cholesterol acyltransferase (ACAT) activity was also observed in cholesterol fed animals (Fig 4.1). The increase, however was much less in RSP fed mice than cellulose fed mice. A trend towards increase in free cholesterol levels due to cholesterol enrichment of the diets was also evident but they rose significantly only in animals maintained on RSP diets. Feeding a diet containing 5% RSP for 4 weeks along with dietary cholesterol also resulted in 50% less increase in hepatic triglyceride levels as compared to cellulose fiber (Table 4.4). In mice fed diets without the added cholesterol, hepatic concentrations of total cholesterol, cholesteryl esters, triglycerides and the activity of ACAT were not affected by the fiber source.

**Table 4.3** ***Effect of dietary RSP (5%) on the plasma lipid levels in mice fed diets with or without supplemental cholesterol (0.5%) for four weeks.***[1,2]

| Diets | Total-chol | TG | LDL-chol | HDL-chol | Free-chol | CE |
|---|---|---|---|---|---|---|
| | | | mmol/l | | | |
| Cellu | $2.12\pm0.02^{a}$ | $0.62\pm0.06^{a}$ | $0.24\pm0.03^{a}$ | $1.60\pm0.02^{a}$ | $0.41\pm0.02^{a}$ | $1.71\pm0.01^{a}$ |
| RSP | $2.22\pm0.05^{a}$ | $0.63\pm0.05^{a}$ | $0.26\pm0.04^{a}$ | $1.69\pm0.03^{a}$ | $0.44\pm0.02^{a}$ | $1.72\pm0.01^{a}$ |
| Cellu + Chol | $2.03\pm0.08^{a}$ | $0.55\pm0.09^{a}$ | $0.45\pm0.05^{b}$ | $1.36\pm0.07^{b}$ | $0.38\pm0.02^{ab}$ | $1.65\pm0.01^{a}$ |
| RSP+ Chol | $1.77\pm.06^{b}$ | $0.50\pm0.03^{a}$ | $0.31\pm0.04^{a}$ | $1.23\pm0.02^{b}$ | $0.33\pm0.01^{b}$ | $1.44\pm0.01^{b}$ |

[1] Values are means ± SEM of six animals

[2] In each column values not sharing a common superscript letter are significantly different at $p<0.05$

Cellu: Cellulose; RSP: Rhubarb Stalk Powder; Chol: Cholesterol; TG: Triglycerides ; CE: Cholesteryl esters.

**Table 4.4** ***Effect of dietary RSP (5%) on the liver lipid levels in mice fed diets with or without supplemental cholesterol (0.5%) for four weeks***$^{1,2}$

| Diets | Total-chol (μmol/g) | TG (μmol/g) | Free-chol (μmol/g) | CE (μmol/g) |
|---|---|---|---|---|
| Cellu | 8.05 ± 0.53$^{a}$ | 13.74 ± 2.82$^{a}$ | 2.84 ± 0.55$^{a}$ | 5.87 ± 0.47$^{a}$ |
| RSP | 7.31± 0.66$^{a}$ | 18.85 ± 2.18$^{a}$ | 2.73 ± 0.24$^{a}$ | 4.57 ± 0.72$^{a}$ |
| Cellu + Chol | 48.70±4.43$^{b}$ | 70.68±7.09$^{b}$ | 3.15 ± 0.09$^{ab}$ | 41.13 ± 4.99$^{b}$ |
| RSP + Chol | 32.32± 3.36$^{c}$ | 46.61 ± 6.08$^{c}$ | 3.54 ± 0.17$^{b}$ | 27.07 ± 5.44$^{c}$ |

[1] Values are means ± SEM of six animals.
[2] In each column values not sharing a common superscript letter are significantly different at $p < 0.05$.
Cellu: Cellulose; RSP: Rhubarb Stalk Powder; Chol: Cholesterol ; TG: Triglycerides ; CE: Cholesteryl esters; ACAT: Acyl CoA : cholesterol acyltransferase

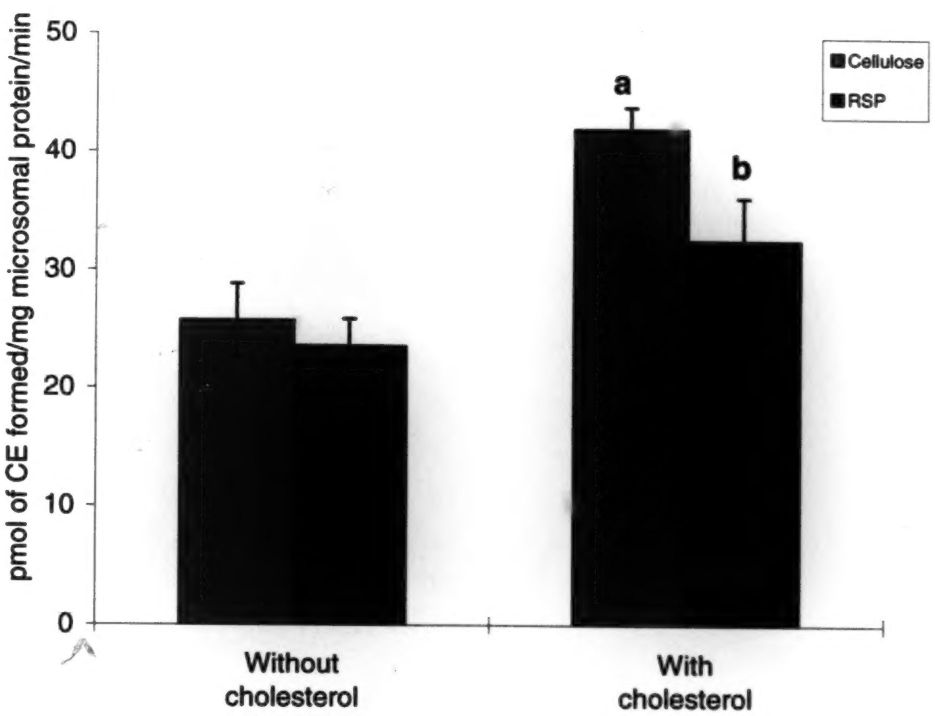

**Fig 4.1 Effect of dietary RSP (5%) on the activity of ACAT in the liver in mice fed diets with or without supplemental cholesterol (0.5%) for 4 weeks.**

Values are means ± SEM of six animals. Bars not sharing a common superscript letter are significantly different at $p<0.05$.

### *4.3.3 Effect of RSP on biliary lipids*

Since, the liver maintains whole body cholesterol homeostasis through secretion of cholesterol and bile acids into the bile, we studied the effects of RSP on the biliary concentrations of these steroids when given with or without dietary cholesterol. Mice fed diets enriched with cholesterol had significantly higher concentration of total cholesterol in gall bladder bile as compared to control mice (Table 4.5). However, no differences were observed between the two fiber sources. Biliary total bile acid concentrations also tended to increase in mice fed cholesterol enriched diets, although the increase was significant only in cellulose fed hypercholesterolemic animals. Mice maintained on RSP displayed lower concentrations of biliary bile acids as compared to cellulose fed animals, although the levels were significantly lower in animals fed diets without supplemental cholesterol.

**Table 4.5** ***Effect of dietary RSP (5%) on the biliary concentrations of total cholesterol and total bile acids in mice fed diets with or without supplemental cholesterol (0.5%) for four weeks*** [1,2]

| Diets | Total- chol (mmol/l) | Total Bile acids (mmol/l) |
|---|---|---|
| Cellu | 4.52 ± 0.36[a] | 68.51 ± 5.55[a] |
| RSP | 2.90 ± 0.58[a] | 39.39 ± 3.98[b] |
| Cellu +Chol | 14.59 ± 1.64[b] | 93.92 ± 12.05[c] |
| RSP + Chol | 12.14 ± 0.29[b] | 82.88 ± 7.31[ac] |

[1] Values are means ± SEM of six animals.

[2] In each column values not sharing a common superscript letter are significantly different at $p<0.05$. Cellu: cellulose ; RSP: Rhubarb ; Chol: cholesterol

### *4.3.4 Effect of RSP on fecal lipids*

Feeding RSP or cellulose fiber diets with or without supplemental cholesterol had no effect on fecal cholesterol excretion (Table 4.6). However, fecal bile acid levels were influenced both by the cholesterol content as well as the choice of fiber present in the diets. Cholesterol enrichment of the diets caused approximately 2.5 fold increase in the fecal bile acid levels. RSP fed animals showed a trend of increased fecal bile acids and the effects became significant ($p<0.05$) when RSP was fed along with dietary cholesterol.

**Table 4.6** ***Effect of dietary RSP or cellulose (5%) on the fecal cholesterol and bile acid levels in mice fed diets with or without supplemental cholesterol (0.5%) for four weeks*** [1,2]

| Diets | Total-Chol (μmol/day) | Bile acids (μmol/day) |
|---|---|---|
| Cellu | 20.40± 5.01[a] | 0.44 ± 0.08[a] |
| RSP | 26.93 ± 2.80[a] | 0.62 ± 0.06[a] |
| Cellu + Chol | 29.02 ± 3.25[a] | 1.20 ± 0.08[b] |
| RSP + Chol | 29.70 ± 3.02[a] | 1.61 ± 0.21[c] |

[1] Values are means ± SEM of six animals.

[2] In each column values not sharing a common superscript letter are significantly different at $p<0.05$ Cellu: cellulose; RSP: Rhubarb Stalk Powder; Chol: cholesterol

### *4.3.5 Effect of RSP on cholesterol 7α-hydroxylase activity and mRNA abundance*

The accelerating effect of RSP on fecal bile acid excretion in combination with its depressing effect on biliary bile acids, led us to examine its effects on the activity of hepatic microsomal cholesterol 7α-hydroxylase and its mRNA abundance. When fed cholesterol rich diets, the activity of 7α-hydroxylase increased significantly in both fiber groups. It was, however, of interest to note that the activity was further increased significantly ($p<0.05$) in RSP fed hypercholesterolemic animals than cellulose fed hypercholesterolemic mice (Fig 4.2).

The dietary induced changes in cholesterol 7α-hydroxylase activity were accompanied by parallel changes in mRNA levels. The relative abundance of cholesterol 7α-hydroxylase mRNA was increased when mice were fed cholesterol-rich diets and the animals fed RSP along with dietary cholesterol expressed the highest levels (Fig 4.3).

## *4.4 DISCUSSION*

Removal of excess cholesterol from the body is accomplished by secretion of cholesterol either directly into the bile or after conversion to bile salts in the liver (Russel & Setchell, 1992). The biliary bile acids are released into the intestine from where they could be either excreted into the feces or are reabsorbed either passively or actively through ileal bile acid transporters then recirculated to hepatocytes through portal circulation. Anion exchange resins and bile acid sequestrants such as cholestyramine and colestipol and inhibitors of ileal bile acid transporters such as compound 2164U90 have been shown to increase fecal bile acid excretion and are associated with a concomitant reduction in biliary bile acid pool (Lewis et al. 1995). The interruption of enterohepatic bile acid circulation, thereby leads to increased diversion of cholesterol to bile acid synthesis in the liver, upregulation of lipoprotein receptors and depressed plasma cholesterol concentrations (Russel & Setchell, 1992). The reductions of up to 25 to 35% in plasma LDL-cholesterol have been reported with these resins (Levy, 1984). However, because the resins often exhibit hypertriglyceridemic effects by decreasing the VLDL catabolism (Gaw et al, 1996). they are limited to use in patients with type-II hyperlipidemia (Ast & Frishman, 1990).

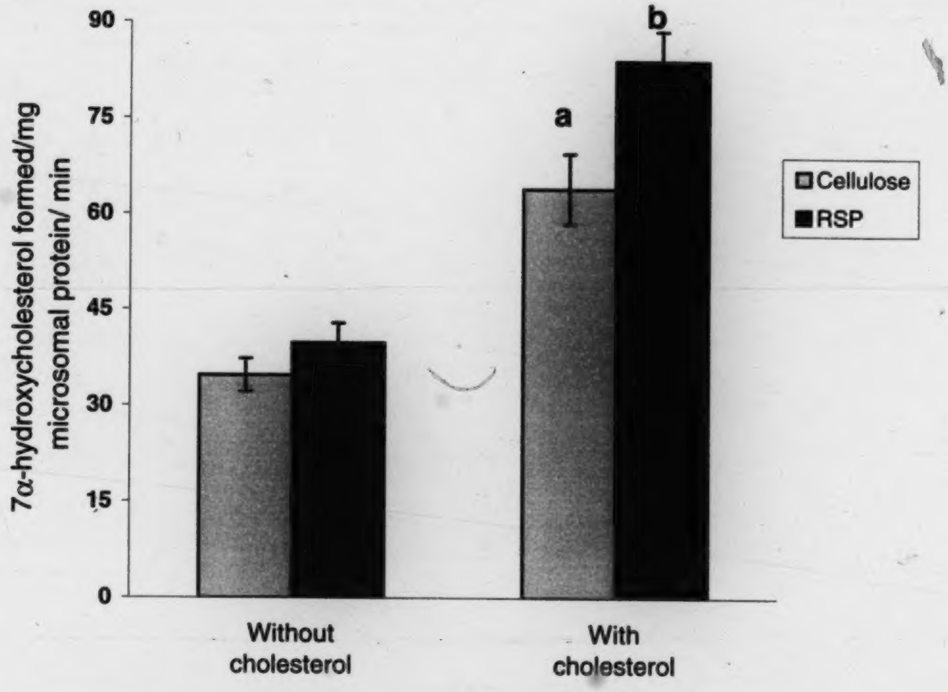

**Fig 4.2 Effect of dietary RSP on the activity of cholesterol 7 α-hydroxylase in mice fed diets with or without supplemental cholesterol for 4 weeks.**

Values are means ± SEM of six animals. Bars not sharing a common superscript letter are significantly different at $p<0.05$.

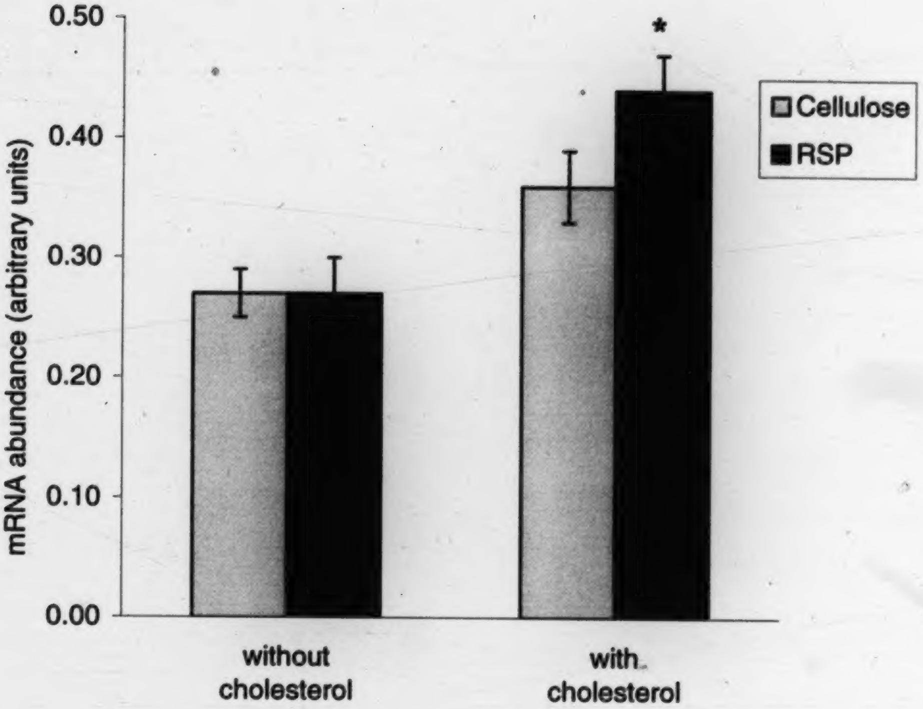

**Fig 4.3 Effect of dietary RSP on the mRNA abundance of cholesterol 7α-hydroxylase in mice fed diets with or without supplemental cholesterol (0.5%) for 4 weeks.** Values are means ± SEM of 6 animals. *P<0.05

Additionally, because of undesirable side effects associated with their continuous use and large doses often required to bring significant reduction in plasma cholesterol levels, resin therapy is considered secondary or an adjunct to dietary modifications (Gamble, 1994).The recommended dietary modifications in the treatment of hyperlipidemia include lowering the fat and increasing the dietary fiber content (Goodman, 1988).

Many fibers and related compounds such as psyllium, pectin and resistant starch have proved very effective in this regard (Fernandez, 1995 ; Younes et al. 1995). Although the underlying mechanisms of the hypocholesterolemic action of these fiber sources are still incompletely understood, the capacity to trap sterols and bile acids (and therefore mimic ion exchange resins) is an important feature of a fiber in depressing plasma cholesterol concentrations (Moundras et al. 1994). The other desirable properties of the fibers include the fermentability, which can produce propionic acid (an inhibitor of HMG-CoA reductase) and viscosity which interferes with dietary cholesterol absorption (Moundras et al. 1994).

Both clinical and experimental trials have indicated that RSP is a novel hypocholesterolemic agent (Basu et al. 1993). In line with the previous observations, the cholesterol - lowering effect of RSP in the present study was evident when fed with a high cholesterol diet and was centered primarily on LDL-cholesterol content. Other animal studies have also shown more pronounced cholesterol lowering effects of dietary fiber in the presence of high doses of dietary cholesterol (Nishina & Freedland,1990; Fernandez et al. 1994). In accordance with the data presented here, most studies have found no changes in plasma triglyceride concentrations with dietary fiber treatment (McCall et al.1992; Fernandez et al. 1994). Significant reductions in hepatic total-cholesterol and cholesteryl-ester concentrations were also achieved with RSP in cholesterol fed animals. Nonetheless, the fiber also elicited hepatic triglyceride lowering effects in these animals.

The present study was conducted to determine a possible mechanism for the observed hypocholesterolemic effects associated with RSP intake. Since the major portion of RSP is  insoluble fiber and fiber elicited only moderate fermentability (Chapter-3), it is unlikely that its cholesterol lowering effects is mediated predominantly through interference of lipid absorption, micelle formation due to viscosity, or via the fermentation products such as short chain fatty acids. Previous investigations have shown that an elevated excretion of bile salts was necessary to obtain a significant cholesterol lowering effect with dietary fibers (Moundras et al. 1994). In accordance with these observations RSP feeding was associated with increased fecal losses of bile salts and a decreased concentration of biliary bile salts. This effect is likely related to the bile salt binding ability of the fiber, as has been shown in an accompanying *in vitro* study (Chapter 4). Although some studies have indicated that bile acid binding or entrapment capacity of non-viscous and nonfermentable fibers was not sufficient to lower plasma cholesterol concentrations (Zacour et al. 1992), RSP also contains soluble fiber, mainly as pectin, and its moderate fermentability (Chapter 1) might have served as additive factors contributing to its effectiveness in lowering plasma lipids.

In RSP fed mice the increased fecal losses of bile salts and diminished gall bladder bile salt concentration were accompanied by increased mRNA abundance as well as the activity of cholesterol 7α-hydroxylase. Recent reports have indicated that cholesterol 7α-hydroxylase besides being regulated by dietary cholesterol and bile acids returning to the liver through portal circulation, dietary factors such as fiber like psyllium (Horton et al.1994) and dietary fats (Cheema et al.1997) can also influence the enzyme activity.

As expected cholesterol enrichment of the diets in this study caused a significant increase in the activity of ACAT (acyl CoA : cholesterol acyltransferase), the enzyme that catalyzes the conversion of free cholesterol to cholesteryl esters in the tissues (Grogan et al. 1991). The increase, however, was much more pronounced in cellulose group as compared to RSP group, leading to a greater accumulation of cholesteryl esters in the livers of mice fed cellulose along with cholesterol. The increased bile salt excretion due to RSP feeding might have increased the conversion of cholesterol to bile salts, consequently decreasing the formation of cholesteryl esters and resulting in a reduction of the concentrations of total and in particular, esterified cholesterol in the liver.

The liver compensates for the loss of cholesterol either by increasing the rate of de novo synthesis or by upregulating the hepatic LDL-receptor (Dietschyl et al., 1993). Though the direct measurement of the LDL-receptor was not made in this study, previous data (Basu et al., 1993) has indicated that RSP does not cause an upregulation of hepatic HMG-CoA reductase activity despite its hepatic cholesterol lowering effects in cholesterol fed mice. These results suggest that LDL-receptor might be the major compensatory pathway for accelerated cholesterol loss in RSP fed mice. It is thus possible that this receptor mediated uptake may be responsible for lowering of plasma LDL-cholesterol levels. Some other fibers such as pectin have also been shown to cause an upregulation of LDL-receptor and cause significant reductions in plasma LDL-cholesterol concentrations (Fernandez et al., 1992).

In conclusion, this study shows that RSP stimulates the reduction of plasma total and LDL-cholesterol concentrations in cholesterol fed mice. This effect is likely due to improved bile salt synthesis and excretion. The results suggest that RSP could be a valuable dietary supplement in the management of mild to moderate hyperlipidemia. Unlike the synthetic resins, RSP did not exhibit any hypertriglyceridemic effects and our reports from clinical trial have indicated that it was well tolerated by the subjects.

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# CHAPTER 5

## Effects of RSP on the bioavailability of calcium in rats[1]

### *5.1 INTRODUCTION*

Experimental as well as clinical trials (Chapter-2 and Chapter-4) have indicated that rhubarb stalk powder, prepared by blanching, drying and grinding the fresh stalks of rhubarb plants is a potential source of dietary fiber for the management of mild to moderate hypercholesterolemia. However, RSP contains a considerable amount of oxalic acid (Table 2.1). Some reports have indicated that dietary oxalic acid can form insoluble salts in the intestine by binding with divalent cations such as calcium and thereby it may affect their utilization (Heaney et al., 1988; Heaney & Weaver, 1989; Kelsey & Prather, 1983). There are, however, many conflicting reports as to whether dietary oxalic acid alone or fiber is responsible for affecting calcium absorption (Ismail-Beigi et al., 1977; Slavin & Marlett, 1980; Toma & Curtis, 1986). It is, therefore, important to examine if the high oxalic content of RSP may be a limiting factor for its use. Hence, this study was undertaken to investigate the effects of different levels of RSP intake on the bioavailability of calcium in rats. This study also validated the hypercholesterolemic action of RSP in normocholesterolemic rats.

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## 5.2 MATERIALS AND METHODS

### 5.2.1 *Animals and diets*

Twenty five Sprague-Dawley male weanling rats weighing 80-90 g were randomly divided into five groups. The animals were housed individually in hanging stainless steel cages. The groups received semi-synthetic diets containing 0, 1, 3 or 5% RSP (Table 5.1). Cellulose was added so that each diet contained 5% fiber. A fifth group was fed a diet containing pure oxalic acid (Oxalic acid dihydrate) added in an amount equivalent to that present in 5% RSP and the fiber content was brought to 5% with cellulose. Since RSP contained appreciable amounts of calcium (20.1 mg/g), the calcium levels of all the diets were adjusted to 4.0 mg/g by using calcium free mineral mixture and adjusting the calcium levels by adding calcium chloride.

All animals had free access to their respective diets for 4 weeks. Body weights were recorded once a week. During the fourth week the rats were placed in metabolic cages and for 3 successive days food consumption was measured and feces and urine collected. At the end of 4 weeks, the animals were killed using carbon dioxide chamber. Blood was drawn by cardiac puncture into plastic tubes containing EDTA as an anticoagulant, livers were excised and femur bones were removed. The separated plasma, livers and bones were stored at -40°C until analysis.

### 5.2.2 *Chemical analysis*

Samples of diets, feces and bones were dry ashed. Calcium content in these samples was determined by atomic absorption spectrometry (Willis, 1961).

The bioavailability of calcium was estimated through the calculation of apparent absorption and total retention of calcium.

Apparent Absorption = Total Calcium Intake - Fecal Calcium

Total Retention of Calcium = Total Calcium Intake - (Fecal Calcium + Urinary Calcium)

Oxalic acid content of feces and urine was determined colorimetrically by the method of Hodgkinson and Williams (1972). Briefly, 0.5 ml urine was taken in 20 ml glass tubes. pH was adjusted to 7.0 with 1N NaOH and volume was made to 1.0 ml. 2.0 ml saturated calcium sulfate and 14 ml ethyl alcohol was added and the tubes were kept at room temperature for 24 h. The tubes were then centrifuged at 3000 rpm and the precipitate was dissolved in 2 ml of 2N $H_2SO_4$ and a piece of zinc was added. The tubes were then kept in a boiling water bath till the volume was reduced to 0.5 ml. Then the piece of zinc was removed. The tubes were again boiled for 30 min. Total volume was made to 20 ml with 10 N $H_2SO_4$ and the color was measured at 540 nm. Blank and standards were run simultaneously.

The plasma and liver samples were also analyzed for lipids as described earlier (Chapter-4).

**Table 5.1** *Composition of semi-synthetic diets**

| Ingredients | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Casein | 20 | 20 | 20 | 20 | 20 |
| Corn starch | 64.8 | 63.3 | 63.4 | 63.6 | 64.8 |
| Corn oil | 6 | 6 | 6 | 6 | 6 |
| Vitamin mix† | 1 | 1 | 1 | 1 | 1 |
| Mineral mix†† | 3 | 3 | 3 | 3 | 3 |
| Methionine | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| RSP |  | 1.0 | 3.0 | 5.0 | - |
| Cellulose | 5 | 4.0 | 2.0 | - | 4.6 |
| Calcium chloride | 1.6 | 1.5 | 1.4 | 1.2 | 1.6 |
| Oxalate dihydrate | - | - | - | - | 0.4 |

* Ingredients were from ICN Biochemicals, Cleveland, Ohio, USA.

† AIN Vitamin mix (mg/Kg diet) retinyl acetate 19.8; ergocalciferol 1.38; dl-α-tocopheryl acetate 110; ascorbic acid 495; inositol 55; choline 2227; menadione 24.7; p-aminobenzoic acid 55; niacin 46.7; riboflavin 11; pyridoxin HCl 11; thiamine HCl 11; D-calcium pantothenate 33; biotin 0.2; folic acid 0.99; vitamin b12 0.015.

†† AIN Calcium free mineral mix (%): Potassium phosphate Dibasic 52.81, Monosodium Magnesium Sulfate.7$H_2O$ 8.18, Sodium Chloride 23.13, Ferric Citrate 4.50, Potassium Iodide 0.130, Manganese Sulfate.$H_2O$ 0.741, Zinc Chloride 0.080, Copper Sulfate.5$H_2O$ 0.050, Sodium Selenite 0.001, Chromium Potassium Sulfate 0.055.

### *5.2.3 Statistical analysis*

Statistically significant differences among means were evaluated by ANOVA (one way analysis of variance). Group means were considered to be significantly different at $P<0.05$ as determined by student's t-test (Steel & Torie, 1987).

## *5.3 RESULTS*

Animals fed diets containing 0 to 5% RSP with same calcium content and with supplemental cellulose to adjust dietary fiber level to 5%, exhibited no differences in either food intake or body weight gain (Table 5.2). It was of interest that the fecal weights increased progressively with increase in cellulose content of the diets.

**Table 5.2** ***Average daily food intake, total body weight gain and fecal weight of rats fed diets containing different levels of RSP for 4 weeks***[1,2]

| Diets | Feed intake (g/day) | Body weight gain (g) | Fecal weight (g/day) |
|---|---|---|---|
| 5% Cellu | 17.6 ± 0.6 | 132.0 ± 9.1 | $1.0 \pm 0.1^{ab}$ |
| 1%RSP + 4%Cellu | 17.4 ± 0.8 | 127.4 ± 14.2 | $0.8 \pm 0.1^{b}$ |
| 3%RSP + 2%Cellu | 16.7 ± 0.9 | 132.2 ± 6.9 | $0.7 \pm 0.1^{bc}$ |
| 5% RSP | 17.7 ± 0.7 | 137.4 ± 9.8 | $0.5 \pm 0.05^{c}$ |
| 0.4%Oxalic acid + 4.6% Cellu | 19.3 ± 0.8 | 145.2 ± 8.6 | $1.2 \pm 0.1^{a}$ |

[1] Values are means ± SEM of five animals

[2] In each column values not sharing a common superscript letter are significantly different at $p<0.05$

Cellu: Cellulose; RSP: Rhubarb Stalk Powder.

Table 5.3 presents the results of the calcium balance study. The mean daily calcium intake was similar in all the groups. Although non-significant, there appeared to be a consistent trend that the average daily fecal excretion of calcium fell as the intake of RSP rose. However, the fecal calcium levels were highest in the group fed purified oxalic acid along with cellulose. Thus, in parallel to fecal calcium levels, a trend of increased apparent absorption and total retention of calcium was manifested as the concentration of RSP increased in the diets. The urinary calcium levels were higher in animals fed RSP.

**Table 5.3** ***Apparent absorption and total retention of calcium in rats fed diets containing different levels of RSP for 4 weeks***$^{1,2}$

| Diets | Ca intake (mg/day) | Fecal Ca (mg/day) | App absorption of Ca (mg/day) | Urinary Ca (mg/day) | Total retention of Ca (mg/day) |
|---|---|---|---|---|---|
| 5% Cellu | 69.8 ± 3.4 | 48.8 + 4.5$^{ab}$ | 20.9 ± 4.2 | 0.35 ± 0.2$^{b}$ | 20.7 ± 4.2 |
| 1%RSP + 4%Cellu | 61.8 ± 2.9 | 38.4 ± 3.8$^{b}$ | 23.4 ± 5.6 | 0.98 ± 0.1$^{a}$ | 22.4 ± 5.6 |
| 3%RSP + 2%Cellu | 63.4 ± 3.6 | 35.9 ± 4.7$^{b}$ | 27.5 ± 3.3 | 0.94 ± 0.2$^{ab}$ | 26.6 ± 3.2 |
| 5% RSP | 69.1 ± 3.0 | 34.9 ± 5.8$^{b}$ | 34.8 ±5.8 | 0.60 ±0.1$^{ab}$ | 33.6 ± 5.5 |
| 0.4% Oxalic acid + 4.6%Cellu | 76.8 ±1.6 | 58.1 ± 5.8$^{a}$ | 18.8 ± 6.5 | 0.46 ±0.1$^{ab}$ | 18.3 ± 6.6 |

[1] Values are means ± SEM of five animals

[2] In each column values not sharing a common superscript letter are significantly different at $p<0.05$.

Cellu: Cellulose; RSP: Rhubarb Stalk Powder

The plasma and bone levels of calcium were not affected by any of the diets (Table 5.4). The daily excretion of oxalic acid in urine also remained unaffected by the diets or the oxalic acid supplementation (Table 5.5). The fecal levels of oxalic acid were significantly higher in animals fed dietary oxalate along with cellulose.

**Table 5.4** ***Femur bone and plasma calcium levels of rats fed diets containing different levels of RSP for 4 weeks***[1,2]

| Diets | Femur Ca (mg/dry weight) | Plasma Ca (mg/100ml) |
|---|---|---|
| 5% Cellu | 201.7 ± 2.7 | 18.4 ± 1.5 |
| 1% RSP + 4% Cellu | 201.1 ± 3.5 | 16.4 ± 1.0 |
| 3% RSP + 2% Cellu | 193.5 ± 1.5 | 18.4 ± 0.9 |
| 5% RSP | 196.6 ± 6.7 | 15.4 ± 1.3 |
| 0.4% oxalic acid + 4.6% Cellu | 202.1 ± 3.3 | 18.7 ± 1.1 |

[1] Values are means ± SEM of six animals.

[2] Values were non-significant at $p<0.05$.

Cellu: Cellulose; RSP: Rhubarb Stalk Powder.

**Table 5.5** ***Oxalic acid excretion in urine and feces of animals fed diets containing different levels of RSP for 4 weeks***[1,2]

| Diets | Urinary oxalic acid | Fecal oxalic acid |
|---|---|---|
| | (mg/day) | |
| 5% Cellu | 0.37 ± 0.03 | 0.66 ± 0.30$^b$ |
| 1% RSP + 4% Cellu | 0.36 ± 0.01 | 1.67 ± 0.60$^b$ |
| 3% RSP + 2% Cellu | 0.52 ± 0.04 | 1.90 ± 0.40$^b$ |
| 5% RSP | 0.49 ± 0.04 | 1.50 ± 0.30$^b$ |
| 0.4% Oxalic acid + 4.6% Cellu | 0.52 ± 0.09 | 3.96 ± 1.00$^a$ |

[1] Values are means ± SEM of five animals

[2] In each column values not sharing a common superscript letter are significantly different at $p<0.05$.

Cellu: Cellulose; RSP: Rhubarb Stalk Powder

The lipid responses of the rats fed diets containing 5% cellulose or 5% RSP were also examined. Significantly lower levels of plasma cholesterol with a trend of lowering LDL-cholesterol and triglyceride concentrations was observed in animals fed diets containing 5% RSP as compared to rats fed 5% cellulose (Fig 5.1). No differences could be observed in hepatic concentrations to total cholesterol and triglycerides in any of the groups (Table 5.6).

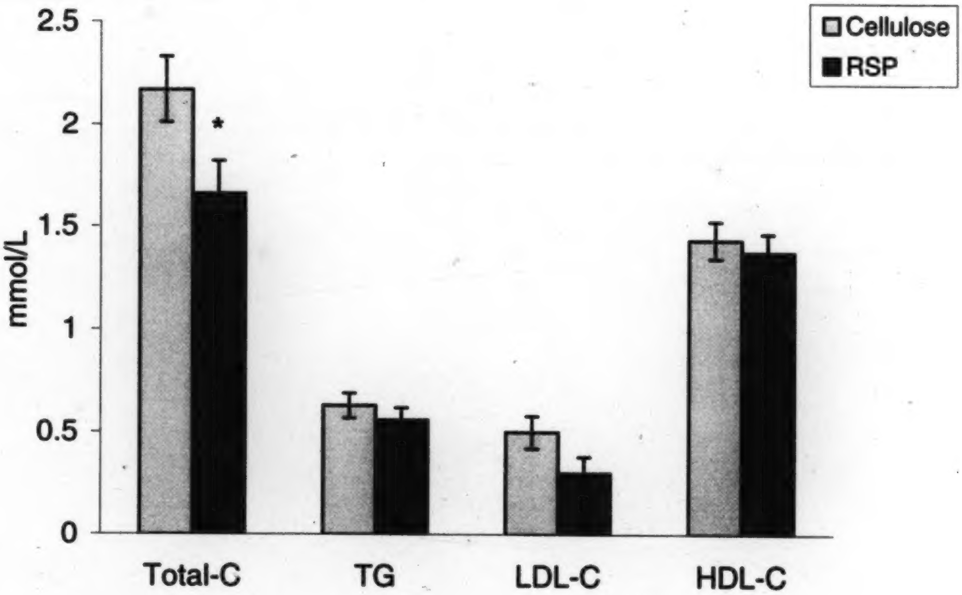

**Fig 5.1 Effect of dietary RSP (5%) for 4 weeks on the plasma lipid levels in normal rats.** Values are means ± SEM of 6 rats. *P<0.05.

**Table 5.6** ***Effect of Dietary RSP (5%) for 4 weeks on the liver lipid levels in normal rats***[1,2].

| Diets | Total-Chol | TG |
|---|---|---|
| | (μmol/g) | |
| 5% RSP | 20.66 ± 3.89 | 20.79 ± 4.01 |
| 5% Cellu | 22.52 ± 2.61 | 27.65 ± 2.62 |

[1] Values are means ± SEM of 6 rats.
[2] Values were non-significant at $p<0.05$.
RSP : Rhubarb Stalk Powder; Cellu : Cellulose; Chol : Cholesterol; TG: Triglycerides.

## *5.4 DISCUSSION*

Since RSP contains considerable amounts of oxalic acid, its effects on calcium bioavailability were examined in rats. The different levels of RSP affected calcium absorption and retention. As the dietary RSP content rose (and cellulose fell), the fecal calcium content fell. The results suggest that all of the calcium in RSP is not bound, or, if it is bound , it was made available by the oxalate degrading microbes in the gut (Argenzio et al., 1988). Nonetheless, synthetic oxalic acid does appear to inhibit calcium absorption. This is indicated by the fact that the group given oxalic acid with cellulose, despite having the highest calcium intake, had the lowest level of absorption and retention of calcium. However, no differences could be observed in bone and plasma calcium levels. This might be because, the animals were still in positive calcium balance and the study was of short duration (4 weeks). The discrepancy in effects between synthetic oxalate and that naturally present in RSP might be related to the type of fiber accompanying it in the diet, since it has been reported that increasing the intake of fiber from cellulose (Slavin & Marlett, 1980; Mod et al., 1985), wheat bran (Tizzani et al., 1989; Watkin et al., 1992; O'Brien et al., 1993), and rice hemicellulose (Mod et al., 1985) causes an increase in fecal weight, fecal calcium and a reduced calcium balance. Thus, it appears that the insoluble fiber fraction in RSP is not predominantly cellulose or hemicellulose. The likelihood of soluble portion of RSP (8%), mainly as pectin, responsible for these effects also cannot be ignored, since fermentable fibers are known to facilitate the solubilization of bound calcium in the colon and therefore favor its uptake by the colonic mucosa (Trinidad et al., 1996). Some reports have indicated that colonic bile salts inhibit the growth of oxalate degrading bacteria (Argenzio et al., 1988). Pectin, due to its ability to accelerate fecal bile salt loss (Garcia-Diez et al., 1996), might have favored the growth of these microorganisms and therefore promoted calcium absorption.

It is, therefore, conceivable that the observation made of the effect of oxalate added to a cellulose containing diet on calcium bioavailability could be the reflection of cellulose intake than oxalate intake. It is also plausible that the dose related increase of RSP on calcium bioavailability could have been caused by decreasing amounts of cellulose present in the diet. However, the comparison of 5% cellulose and 5% RSP group does clearly reveal that RSP did not have any detrimental effect on calcium bioavailability up to its 5% level in the diet.

Unlike, no effects of RSP on plasma cholesterol concentrations in normal mice (Chapter-4), in normal rats, RSP feeding was accompanied by a significant decrease in plasma cholesterol concentrations. The effects might be due to the variation in animal specie and signify the potential preventive as well as therapeutic ability of RSP to combat hypercholesterolemia, though detailed studies to test this hypothesis are still required.

In conclusion, RSP appears to be a valuable source of dietary fiber for the management of hypercholesterolemia The amount of oxalate present in RSP up to its 5% dietary level does not interfere with calcium bioavailability, therefore should not be considered a factor against using this novel fiber source either directly or in preparation of food products.

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# CHAPTER 6

## General discussion and conclusions

Coronary heart disease is not only the major cause of death in the leading industrialized countries, but it accounts for enormous cost for treatment and care. High serum cholesterol, recognized as a significant factor for heart disease, is reversible by diet in many cases. Diet recommendations made for these people include reductions in body weight if necessary; restriction of total lipid intake to < 30% of total calories, saturated fat intake to < 10% calories and cholesterol intake to < 300 mg/day; and increasing the intake of dietary fiber (Goodman, 1988). Consumers today are concerned with health promoting foods, and this trend is growing. The U.S. National Cholesterol Education Program has a primary objective of making the public more conscious of the importance of maintaining normal serum cholesterol concentrations. Thus, the food industry is searching for alternative economical sources of dietary fiber.

Rhubarb, a cold season, hardy crop is vastly underutilized in Canada. Recent evidence indicate that its stalks could be a potential source of dietary fiber. A simple extraction procedure involving blanching, drying and grinding of fresh rhubarb stalks provided a product that was very rich in dietary fiber, 74% on dry weight basis with 66% insoluble and 8% soluble fiber. This rhubarb stalk powder (RSP) elicited a high water holding capacity adsorbing up to 19 times its weight of water, thus has attracted attention of food scientists as a potential filler or bulking agent in food products. Nutritional studies were also undertaken to evaluate the potential ability of RSP as a dietary supplement for health benefits. *In vitro* fermentation of RSP using human fecal microbiota revealed that the fiber source had moderate fermentability, in between those of highly fermentable fibers such as pectin and less fermentable fibers such as cellulose (Unpublished observation).The potential ability of RSP as a hypocholesterolemic agent was also tested in experimental mice. Its dietary supplementation at 5% level in semi-synthetic diets of hypercholesterolemic mice resulted in significant reductions of both plasma and liver cholesterol concentrations (Basu et al., 1993). RSP also led to an increase in HDL to total cholesterol ratio in parallel with significant reductions in plasma and liver triglyceride concentrations. These intriguing, but preliminary results prompted the present study to further explore the potential ability of RSP as a hypocholesterolemic agent. A series of experiments were thus conducted to examine its cholesterol - lowering effects in humans and experimental animals.

C57BL/6J mice are considered to be sensitive to diet induced hypercholesterolemia (Paigen et al., 1987). Using this strain of mice the hypocholesterolemic action of RSP was examined. The animals were fed semi-synthetic diets with or without 0.5% supplemental cholesterol containing 5% RSP or cellulose. Olive oil was used as the fat source in the diets because of the greater ability of monounsaturated fatty acids to produce hypercholesterolemia in the presence of dietary cholesterol (Cheema et al., 1997). Mice fed 5% RSP diets exhibited significantly lower concentrations of plasma and liver cholesterol than the cellulose controls. The effects were more pronounced in mice fed a diet supplemented with 0.5% cholesterol. These results are in agreement with the previous study involving ICR albino mice (Basu et al., 1993), depicting more significant cholesterol-lowering effect of dietary RSP in the presence of high doses of dietary cholesterol. It was noteworthy that the cholesterol-lowering response of RSP was specific for LDL-fraction and that the depressed LDL-cholesterol was accompanied by a decreased hepatic triglyceride level, especially in mice fed a cholesterol-enriched diet.

The cholesterol-lowering effects of RSP were subsequently evaluated in a selected group of hypercholesterolemic subjects. A daily dietary supplement of 27 g of RSP providing 20 g of dietary fiber was ingested by 10 free living men having mild to moderate hypercholesterolemia for 4 weeks. The RSP intervention revealed results similar to that in experimental animals. The total cholesterol and its LDL fraction in plasma were, thus, significantly reduced following the intake of 27 g of RSP/day for 4 weeks, the average reduction of cholesterol levels was 8%. It was noteworthy that all the 10 participants responded to RSP. It was of further interest, that following a wash out period of 4 weeks, the depressed serum cholesterol concentrations of the participants rose again and approached the baseline levels. Although, the baseline values of the subjects were established by averaging two fasting blood samples, the final and washout effects were evaluated through a single sample. It may be argued that since blood cholesterol is subject to a day to day variation (Truswell, 1995), reliability of the cholesterol value in the subjects may be questionable. There are, however, several trends in the present study pointing to the fact that RSP - associated depressed plasma cholesterol level is a true reflection of the fiber intake. These trends include : a) the baseline cholesterol level was similar to the level found at the end of the washout period; b) no appreciable change in dietary intakes with the exception of fiber was observed between the onset of the study and at the end of RSP intervention; c) all study subjects displayed a declining trend in plasma cholesterol level following RSP intervention; d) the hypocholesterolemic action of RSP is similar in both humans and experimental animals.

Having established the hypocholesterolemic action of RSP, the present study was extended to elucidate the underlying mechanism(s) of the action. Multiple mechanisms have been suggested in the cholesterol-lowering effects of dietary fibers, depending on their physiochemical properties which include viscosity, fermentability and binding ability. It has been suggested that soluble fibers by increasing the viscosity of intestinal contents may slow down the diffusion of lipid containing micelles across the intestinal mucosa and therefore may slow down the lipid absorption (Gallaher et al., 1993). Short chain fatty acids produced by fermentation of dietary fibers have also been implicated in the hypocholesterolemic effects, especially propionate due to its ability to inhibit hepatic sterol synthesis (Kishimoto et al., 1995). Some fibers also elicit the ability to bind or sequester bile salts, therefore by causing the interference with the enterohepatic recirculation of bile salts increase fecal bile salt excretion (Fernandez, 1995; Matheson et al., 1995).

Since, RSP was predominantly an insoluble fiber source, it was unlikely that viscosity of the fiber could have been the major attribute responsible for its hypocholesterolemic effects. Also, the new fiber source elicited only moderate fermentability (50% that of pectin), thus limiting the involvement of short chain fatty acids in the observed effects. Therefore, in an effort to elucidate the possible mechanism of cholesterol-lowering effects of RSP an *in vitro* study was carried out, examining its binding capacity with bile salts. According to this study, RSP was found to have a strong ability to bind taurocholic acid and cholic acid. This property of RSP was compared with a number of mixed and insoluble fibers (for example: wheat bran, rice bran, corn bran and cellulose) and also with colestipol, an anion exchange resin and bile acid sequestrant which is used to treat hypercholesterolemia. It was noteworthy that the binding capacity of RSP was second only to colestipol and it bound 11 and 2.5 fold more bile salt than cellulose or wheat bran respectively. According to the competitive binding assay between conjugated taurocholate and unconjugated cholic acid, the conjugation of bile salt does not appear to be essential for the binding to RSP. The fiber has high water-holding capacity, adsorbing up to 19 times its weight of water, thus it is possible that it could have led to entrapment of bile salts in the interstitial spaces of the fiber matrix. It is also possible that the bile salt is entrapped in the gelatinous mass formed by the pectin in RSP. The bile salt adsorption capacity of lignin is well established (Gallaher & Schneeman, 1986). It is likely that RSP contains appreciable amounts of lignin in the insoluble fraction of its fiber. The contribution of calcium fraction of RSP to its novel ability to sequester bile salts also cannot be ignored, since calcium is known to bind bile salts (Govers et al., 1996).

In agreement with the ability of RSP to sequester bile salts *in vitro,* the fiber intake was found to promote fecal loss of bile salt in both normocholesterolemic as well as hypercholesterolemic animals. The increased losses of bile acids were accompanied by reduced the concentration of bile salts in the gall bladder bile. These results may be a reflection of an increased degradation of cholesterol as indicated by an increased activity of the hepatic microsomal cholesterol 7α-hydroxylase. This was supported by an abundant mRNA for the enzyme in hypocholesterolemic animals.

The cholesterol balance in the cell is known to be regulated by the content of free cholesterol which is also known as 'metabolically available pool' of cholesterol (Brown & Goldstein, 1986). It does it by altering the uptake through LDL-receptor, affecting cholesterogenesis by regulating the activity of HMG-CoA reductase, controlling the esterification by enzyme ACAT and in hepatocytes affecting degradation of cholesterol to bile acids. It was interesting to note that the hypercholesterolemic mice fed RSP exhibited higher levels of free cholesterol along with significantly lower activity of ACAT as compared to corresponding cellulose controls. These results correlated with increased activity of hepatic cholesterol 7α-hydroxylase in RSP fed hypercholesterolemic mice.

The liver compensates for the loss of cholesterol by either increasing the rate of de novo synthesis or by upregulating hepatic LDL-receptor activity (Dietschyl et al., 1993). Since the preliminary report by Basu et al. (1993) indicated that RSP does not increase hepatic cholesterol synthesis, suggesting that perhaps LDL-receptor might had been a compensatory pathway and this could have also contributed to lowering of plasma LDL-cholesterol concentrations. Figure 6.1 illustrates the possible suggested mechanism of hypocholesterolemic action of dietary RSP.

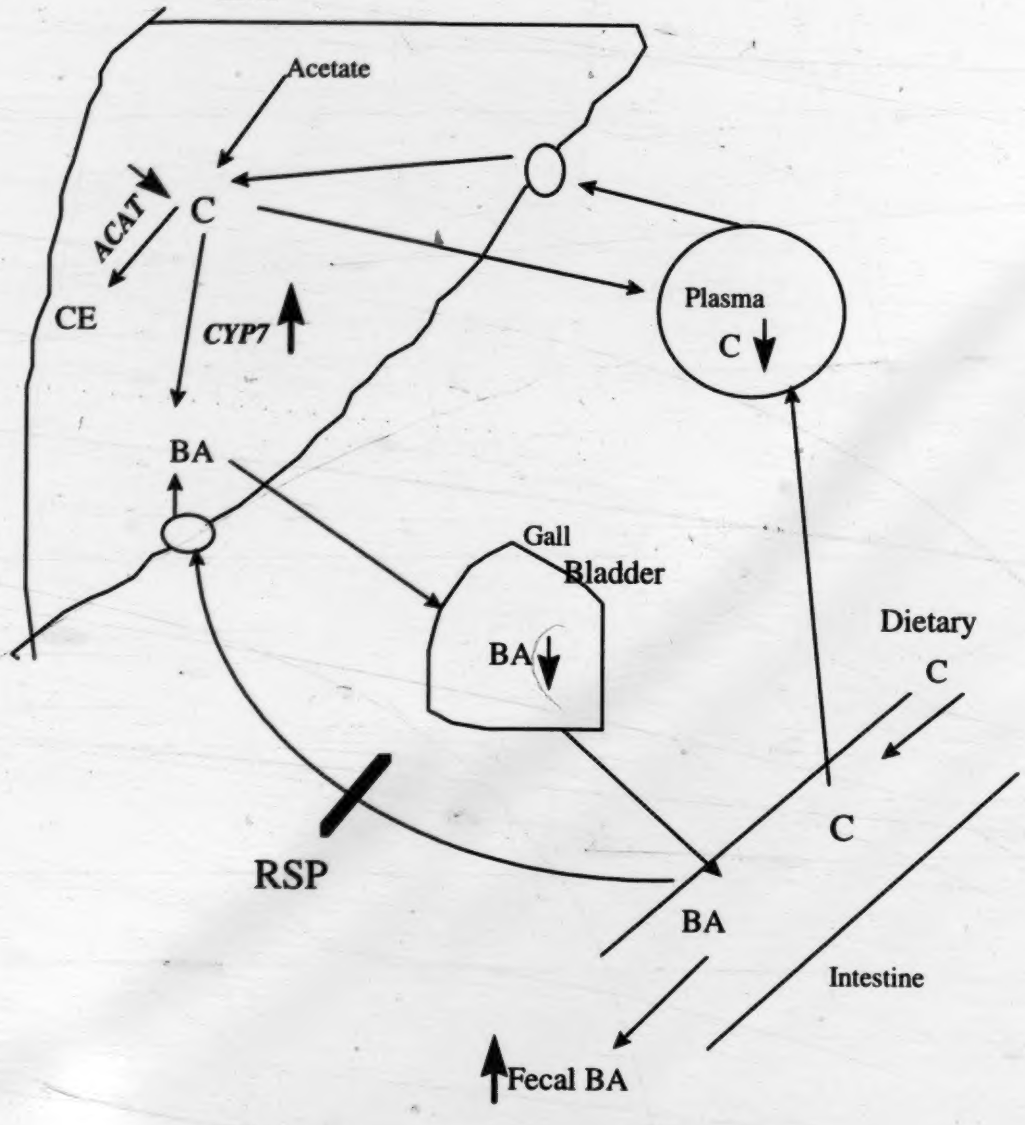
Liver
Acetate
ACAT
C
CE
CYP7
BA
Plasma
C
Gall
Bladder
BA
Dietary
C
C
RSP
BA
Intestine
Fecal BA

Fig 6.1 Proposed mechanism for the cholesterol lowering action of RSP C:cholesterol; CE:cholesteryl ester; BA:bile acids;ACAT: Acyl CoA:cholesterol acyl transferases; CYP7: cholesterol 7 α-hydroxylase; :increase; :decrease

The evidence gathered from this study point to the fact RSP is a valuable hypocholesterolemic agent. In humans the daily intake of RSP at a level of 27 g can potentially lower blood cholesterol levels. The effectiveness of the fiber could be related to its novel ability to sequester bile salts, though the contribution of other properties such as moderate fermentability, moderate solubility also cannot be precluded.

One of the potential concerns for the use of RSP as a fiber supplement is that it contains considerable amounts of oxalic acid, which is known to affect calcium absorption (Heaney et al., 1988; Heaney & Weaver, 1989). Therefore, the dose-response (0-5%) effects of RSP, on apparent absorption and total retention of calcium were examined through a balance study. Male sprague-dawley rats were used as experimental animals, because of their large size, and therefore making collection of fecal and urine samples, and the measurement of food intakes more convenient. The results revealed no detrimental effect of RSP on calcium bioavailability. These results were further substantiated by the fact that the bone calcium levels of the animals remained unaffected by dietary RSP. It should be pointed out, however, that unlike RSP an equivalent amount of synthetic oxalate in a diet significantly increased fecal losses of calcium. It is plausible, that the effects might had been related to the accompanying fiber in the diet, since increased fecal excretion of calcium with cellulose has been reported (Slavin & Marlett, 1980; Mod et al., 1985) and fermentable fibers are known to facilitate calcium absorption from the colon (Trinidad et al., 1996). The calcium bioavailability from calcium oxalate is also affected by the colonic concentration of oxalate degrading bacteria. Some reports have also indicated a negative association between colonic bile salt concentration and oxalate degrading microflora (Argenzio et al., 1988). Since RSP also elicited many fold greater ability to bind bile salts than cellulose (Chapter-3), it is also plausible that RSP by favoring the growth of these microorganisms might have favored calcium absorption. The results, thus suggest that oxalic acid in RSP should not be considered a factor against using this novel source of dietary fiber, either as a dietary supplement or in the preparation of foodstuffs.

In conclusion, the available evidence not only support the potential value of RSP as a dietary supplement for the management of plasma cholesterol concentrations, but also suggest that a substantial scope exists for the increased production and commercialization of this underutilized crop. This is the first extensive study revealing the biological potential of its fiber. The ability of RSP to lower plasma cholesterol concentrations in hypercholesterolemic subjects and in normocholesterolemic as well as in hypercholesterolemic animals is appreciable. The most important highlight of the study, is perhaps, the elucidation of the underlying mechanism. The stronger ability of RSP to bind bile salts *in vitro* than many fibers tested such as wheat bran, corn bran, cellulose and rice bran is noteworthy. Thus, in parallel the fiber feeding was accompanied by increased fecal losses of bile salts, decreased biliary concentration of bile salts and increased activity of cholesterol 7α-hydroxylase. This points to the fact, that may be the fiber acts as a bile acid sequestrant in the gut and exerts cholesterol-lowering effects by interruption of enterohepatic circulation of bile salts. RSP is also unique in chemical composition. Unlike commonly used fiber supplements i.e., soluble or insoluble, the fiber had a mixed composition and also contained a number of associated components such as calcium and organic acids such as oxalic and malic acid. Role of these acids in the context of hypercholesterolemia has not been investigated. Therefore, future studies could involve a) investigation of possible involvement of the organic acids such as oxalic acid and malic acid in the hypocholesterolemic effects of RSP; b) effects of long-term administration of RSP on blood lipid profiles of hypercholesterolemic and normocholesterolemic subjects; c) determining the potential value of RSP as a supplement to increase fecal bulk and to improve gut motility and intestinal transit time; d) testing the hypoglycemic effects of RSP.

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